BioCloneBot: A Low Cost, Open Source, Automated Liquid Handler

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This is to certify that the thesis prepared

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Abstract

BioCloneBot: A Low Cost, Open Source, Automated Liquid Handler

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Automated liquid handlers are devices for automating synthetic biology experiments by moving volumes in the range of 1μL to 1,000μL depending on the experiment. Most currently available devices utilize a stepper motor-based 3-axis system and linear actuated pump. These devices can be set up to automate hundreds or thousands of manual liquid handling steps. These liquid handling steps are both substantially repetitive and often introduce human error. As such, automation can be used to save time and eliminate human error. In this thesis, we describe a low-cost, open-source, automated liquid handler, the BioCloneBot, designed to automate pick and place DNA assembly experiments. The BioCloneBot consists of off-the-shelf and 3D-printed components. To reduce complexity and reduce sourcing multiple parts, an Ender 3 Pro 3D printer is used for its frame, stepper motors, and limit switches. The result is a highly accurate and precise liquid handler with an intuitive front-end making automation more accessible by lowering the cost to less than $\$1,500$ CAD.

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Chapter 1

Introduction

1.1 Background

Synthetic biology is an emerging field that focuses on studying organisms by modifying their DNA [1]. There are many applications of synthetic biology such as medical diagnostics, pharmaceutical development, and curing genetic-based diseases [2]–[5]. Nearly every synthetic biology experiment, especially in the area of genomics, largely consists of liquid handling manipulations. From the extraction of DNA to the synthesis of custom DNA, each experiment involves moving liquids to and from labware such as tubes, wellplates, and reservoirs. In general, scientists use manual pipettes to move liquids such as DNA, enzymes, and reagents between different labware during each step of the process. While studying an organism, a single scientist may perform thousands of physical manipulations. These scientists end up wasting copious amounts of time performing these tedious and repetitive operations, often resulting in human error. If this error is introduced early on, it can easily propagate to the end resulting in a failed experiment. Depending on the steps required for such experiments, it may take multiple days to repeat the process from start to finish. Automation can save scientists time by performing some or all of these manipulations while reducing human-introduced errors.

While there are numerous protocols used within synthetic biology, DNA assembly protocols are among the key protocols due to their ability to modify and synthesize custom DNA segments and plasmids. A DNA segment refers to a linear piece of DNA whereas a DNA plasmid is a circular piece of DNA. Three of the most commonly used DNA assembly protocols include Restriction Enzyme Digestion and Ligation, Gibson

Assembly, and Golden Gate Cloning. Gibson Assembly and Golden Gate Cloning are powerful due to their ability to assemble multipart constructs in a one-pot reaction [6], [7]. A one-pot reaction occurs when all inputs are mixed into a single tube, and then the same tube is used for each subsequent step.

Before devising an automated solution, it is important to have a high-level understanding of the Restriction Enzyme Digestion & Ligation, Gibson Assembly, and Golden Gate Cloning protocols. These three protocols can be summarized into a combination of two major types of steps: mixing the ingredients into a single tube (liquid handling) and running the tube through a thermocycler (amplification). The goal of this thesis is to develop a low-cost, open-source, easy-to-assemble, and easy-to-use automation device capable of automating the liquid handling portion of these protocols. The following subsections will describe PCR, a necessary step in the DNA assembly process, and the three protocols.

1.1.1 PCR Amplification

PCR amplification is a protocol for creating copies of desired DNA segments [8]. Notably, PCR amplification was one of the primary and most reliable methods of viral detection during the COVID-19 pandemic [9], [10] emphasizing its importance. It is a necessary step performed during nearly all DNA assembly protocols.

There are four major steps to perform a PCR amplification: collection, preparation, amplification, and post-PCR clean-up [11]. During the collection step, samples of the target organism are collected or specific DNA can be ordered from companies such as addgene [12]. Addgene is a DNA repository where researchers can deposit and purchase affordable DNA plasmids containing a specific gene of interest. Once the sample is acquired, the next step is preparation. During preparation, the DNA sample is extracted and purified. The now purified DNA, DNA primers, DNA polymerase, and dNTPs (single nucleotide bases: A, T, C, and G) are mixed into a single tube. The DNA polymerase is an enzyme that synthesizes DNA by extending the 3' end of an annealed (attached) primer. Primers are short, complementary pieces of DNA that anneal to the 3' side of the template strand and act as the start place of the complimentary strand. To synthesize the complimentary strand, the DNA polymerase attaches complimentary bases to the template strand one base at a time. Once everything is sufficiently mixed together, the amplification step occurs. The tube is placed inside a thermocycler, which is a device that performs cycles of precise heating and cooling. During each cycle, the thermocycler performs three steps: *denaturation* where the tube is heated to at least 94°C to break

down the hydrogen bonds and separate the target DNA into its single strand components, *annealing* where the mixture is cooled to a temperature between 50-60°C allowing the DNA primers to anneal to the template strands, and *extension* where the DNA polymerase attaches the dNTPs to the single-stranded DNA forming new double-stranded DNA copies of the target DNA. This process is repeated between 30-40 times doubling the quantity of DNA at an exponential rate.

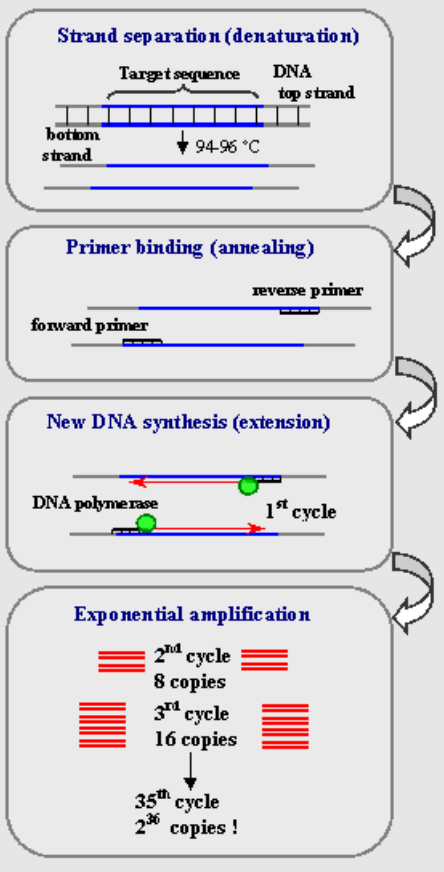


Figure 1.1: **Overview of PCR Amplification step [8].**

Finally, the analysis step is performed. A sample of the resultant DNA and a DNA ladder is run through a gel electrophoresis which provides a confirmation of the PCR amplification. The DNA ladder contains DNA of various sizes. During the gel procedure, an electric field pushes the pieces of DNA through the gel. The smaller the size of DNA, the further distance it travels. An example gel electrophoresis image can be seen in figure 1.2. As a more accurate confirmation, the resultant DNA can be shipped to a company for sequencing.

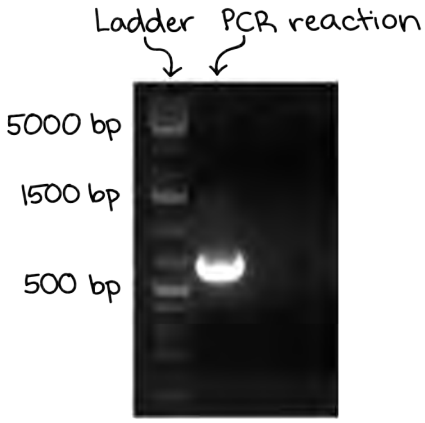


Figure 1.2: **Sample gel electrophoresis [13]. By examining a resultant gel, a confirmation of the PCR amplification can be seen. The gel can verify that the target DNA is approximately the expected length. To verify the full integrity of the DNA, it can be sent for DNA sequencing.**

1.1.2 Restriction Enzyme Digestion & Ligation

Restriction enzyme digestion and ligation allow for the insertion of linear DNA (referred to as the gene of interest) into a DNA plasmid. It utilizes two types of key enzymes: restriction enzymes and DNA ligase enzymes. Restriction enzymes work by targeting specific short sequences of DNA, then digesting (cutting) the DNA into two pieces (or one linear piece in the case of a DNA plasmid). At the cut site, a blunt end or short single-stranded overhang of a few bases remains. These overhangs allow for a new piece of DNA to be connected via the annealing through the complimentary DNA overhangs. DNA ligase acts as the glue allowing for the gene of interest to be inserted into the target DNA plasmid. For restriction enzyme digestion and ligation to work, it is essential for the donor plasmid and the recipient plasmid to have complementary overhanging sequences. This is usually achieved by using a restriction enzyme capable of targeting a site on both plasmids.

Digestion and ligation are the two key steps of the protocol. During the digestion process, DNA, two restriction enzymes, a buffer, BSA, and dH_2O are mixed together into a single tube. Each input ranges between 1μL and 10μL. Once well mixed, the tube is then placed into an incubator at 37°C for at least one hour to allow enough time for the restriction enzyme to digest the DNA segments or plasmids. The result is run through a gel electrophoresis for confirmation and isolation of the purified DNA of interest. To perform the ligation process, the gene of interest, the linearized recipient plasmid, ligase buffer, DNA ligase, and dH_2O are mixed into a tube. This tube is then placed into an incubator for two hours allowing for gene of

interest to successfully ligate to the linearized recipient plasmid. A final gel electrophoresis is performed for confirmation of the final DNA plasmid containing the gene of interest. The resultant DNA plasmid is then transformed into the target organism (generally E. coli) via heat shock or electric shock. Once transformed, successful expression of the plasmid can be observed via a selection marker.

1.1.3 Gibson Assembly

Gibson Assembly is used for the synthesis of custom plasmids containing one or more chosen parts. It can be broken down into three key steps. The first step is to linearize the target vector plasmid. Using 1-2 restriction enzymes, a circular vector plasmid is cut to form a single linear piece of DNA with two blunt ends. The second step is to synthesize linear inserts containing the gene of interest and template specific ends complimentary to the ends of the linearized vector. During this step, special primers are designed that overlap with the gene of interest and the target plasmid. Once run through a PCR amplification, the gene of interest will be extended into a longer piece of linear DNA capable of inserting into the vector plasmid. For a multipart plasmid, this process is repeated where each piece must be designed so that the end of one piece is complimentary to the beginning of the next piece. The third step is to mix the linearized vector plasmid, the extended gene(s) of interest, and a Gibson Assembly master mix into a single tube. This is then incubated at 50°C for between 15 minutes and 1 hour. During this step, an exonuclease chews back the 5' end of the double-stranded DNA allowing for the pieces of DNA to anneal together. Once annealed, a DNA polymerase fills in any missing bases, and then a DNA ligase glues the pieces together at the anneal site resulting in a scar-less, custom DNA plasmid. Gibson Assembly is generally performed with volumes ranging from 5μL to 15μL.

1.1.4 Golden Gate Cloning

Similar to Gibson Assembly, Golden Gate Cloning is used to insert one or more pieces of linear DNA into a single DNA plasmid. To perform this insertion, restriction IIS enzymes such as BsaI are used. As opposed to regular restriction enzymes, IIS restriction enzymes cut outside of their target DNA sequence which is used for the removal of the IIS recognition sites. Generally IIS enzymes that create a 4-base overhang are used which allows for the creation of up to 256 unique 4-base overhangs. Unlike the restriction enzyme and digestion protocol, Golden Gate performs both the digestion and the ligation in a single step. Golden Gate

cloning consists of three steps. The first step is to design a vector plasmid that contains two IIS recognition sites where gene(s) of interest can be inserted after cleavage. The second step is to design a linear gene of interest that has a IIS recognition site on both ends. Note that the gene of interest could exist inside of a plasmid containing two IIS recognition sites. It is crucial that the 4-base overhangs of the gene of interest created by the IIS restriction enzymes are complimentary to the 4-base overhangs of the vector plasmid. For a multipart plasmid, the overhangs must be designed so that the 4-base overhang at the end of one piece of DNA is only complementary to the 4-base overhang at the beginning of the next piece of DNA. The final step is to mix the the target plasmid, the gene(s) of interest, DNA ligase, and the IIS restriction enzymes into a single tube. This tube is placed into thermocycler which alternates temperatures between 16°C and 80°C for digestion, annealing, and ligation. Once the gene(s) of interest and target plasmid are digested leaving 4-base overhangs, the gene of interest anneals to the vector plasmid. The DNA ligase finishes off by gluing all the pieces together forming scar-less plasmids. Golden Gate cloning generally requires the movement of volumes between 1μL and 20μL.

1.2 Literature Review

Before deciding on the key features of the proposed device, it is important to understand the currently available solutions. While there are a plethora of commercially available options, only a few key options can be found in literature. For this review, only all-in-one liquid handlers capable of working with more than one labware are explored. In addition, devices above 50K CAD will not be explored. It is important to note that the prices are not advertised for most commercially available liquid handlers. In the case that a price was not able to be found, the price for a used device is considered.

1.2.1 Eppendorf epMotion 5075t

Figure 1.3: **Eppendorf epMotion 5075TMX owned by the University of Oregon [14].**

The epMotion 5075t is an automated liquid handler made by Eppendorf in 2020. The 5075t features 14.5 deck positions capable of supporting various labware and additional add-on thermal modules. The required software and device hardware are not open-source. The software provides an intuitive platform for creating user-defined experiments. The 5075t also features a gripper capable of moving labware from one deck position to another. Eppendorf sells multiple accessories that can allow the support of a wide array of wellplates and tubes. It also supports multiple reservoirs and thermal blocks for on device heating. An optical sensor is used for contact-free liquid level sensing and labware recognition.

The 5075t supports up to six different dispensing tools. The dispensing tools are automated pipettors used to pick up a sample from a source labware and then dispense the sample into a destination labware. There are both single and 8-channel pipettors for the 1.0-50.0μL, 20.0-300.0μL, and 40-1000μL volume ranges. While it is recommended to use pipette tips designed by Eppendorf, the 5075t may support pipette tips already in use by a lab. According to the epMotion manual, the pipettors work via a piston that utilizes the air-cushion principle. A stepper motor is used to control a piston that aspirates a volume when going up and dispenses a volume when going down [15]. The 5075t includes the capability of automatically swapping between dispensing tools during an experiment. Each dispensing tool supports tip ejection. Specifications for each automated pipettor can be found below in table 1.1.

Dispensing Tool	Volume	Systematic Error	Random Error
TS 50	1μL	$\pm 20.0\%$	$\pm 5.0\%$
TS 50	5μL	$\pm 5.0\%$	$\pm 3.0\%$
TS 50	25μL	$\pm 1.5\%$	$\pm 0.6\%$
TS 50	50μL	$\pm 1.0\%$	$\pm 0.4\%$
TS 300	20μL	$\pm 4.0\%$	$\pm 2.5\%$
TS 300	30μL	$\pm 3.0\%$	$\pm 1.5\%$
TS 300	150μL	$\pm 1.0\%$	$\pm 0.4\%$
TS 300	300μL	$\pm 0.6\%$	$\pm 0.3\%$
TS 1000	40μL	$\pm 5.0\%$	$\pm 1.5\%$
TS 1000	100μL	$\pm 2.0\%$	$\pm 1.0\%$
TS 1000	500μL	$\pm 1.0\%$	$\pm 0.2\%$
TS 1000	1000μL	$\pm 0.7\%$	$\pm 0.15\%$
TS 50_8	1μL	$\pm 25.0\%$	$\pm 10.0\%$
TS 50_8	5μL	$\pm 5.0\%$	$\pm 5.0\%$
TS 50_8	25μL	$\pm 2.0\%$	$\pm 1.2\%$
TS 50_8	50μL	$\pm 1.2\%$	$\pm 0.6\%$
TS 300_8	20μL	$\pm 10.0\%$	$\pm 4.0\%$
TS 300_8	30μL	$\pm 10.0\%$	$\pm 3.5\%$
TS 300_8	150μL	$\pm 2.5\%$	$\pm 0.8\%$
TS 300_8	300μL	$\pm 1.5\%$	$\pm 0.5\%$
TS 1000_8	40μL	$\pm 6.0\%$	$\pm 2.5\%$
TS 1000_8	100μL	$\pm 3.0\%$	$\pm 1.5\%$
TS 1000_8	500μL	$\pm 1.5\%$	$\pm 0.3\%$
TS 1000_8	1000μL	$\pm 0.8\%$	$\pm 0.15\%$

Table 1.1: **Eppendorf epMotion 5075t Pipette Specifications [15]**

The EpMotion is quoted starting at $110,000$ CAD, though it is also available on the used market for as low as $\$25,000$ CAD. The single-channel dispensing tools can be found for around $\$4,000$ CAD each and the eight-channel dispensing tools are sold for around $\$7,200$ CAD each.

1.2.2 Hamilton Microlab NIMBUS4

Figure 1.4: **Hamilton Microlab NIMBUS4 enclosed model [16].**

The Hamilton Microlab NIMBUS is a family of automated liquid handlers sold by the Hamilton company. Each model comes in four different sizes each with the option for a 4-tip, 96-tip, and 384-tip pipettor. For this review, the smallest NIMBUS with the 4-tip pipettor will be reviewed. The NIMBUS4 comes in three different deck configurations: an 11-position configuration featuring nine main positions and two sub-positions, a 3x4 configuration with 12 main positions, and a Shift-n-Scan configuration featuring 8 main positions with an integrated tube bar-code scanner. The device supports volumes between 0.5μL and 1000μL. Hamilton sells an array of accessories including labware pedestals for labware such as tip racks, microtiter plates, tubestands, and more. They also sell adapters to accommodate wellplates, tubes, and tips. Hamilton offers intuitive, easy to use proprietary software for user-created experiments. The device also includes a gripper capable of moving labware to other locations on the experiment deck.

The pipettors are designed using special Hamilton proprietary technology. It features a special o-ring design compatible with CO-RE Hamilton pipette tips for a perfect seal and easy tip ejection. The pipettor utilizes air displacement technology for aspirating and dispensing. It also features liquid level detection, monitored air displacement, and anti-droplet control. The specifications of the NIMBUS4 pipettor can be found in table 1.2 below. The NIMBUS4 features four 1000μL channels, but can support lower volumes depending on the tips used. Hamilton does not advertise if the NIMBUS is compatible with already available

pipette tips.

Tip Size	Volume	Truness	Precision CV
10μ	1μL	$\pm$5.0%	$\pm$5.0%
10μ	5μL	$\pm$2.5%	$\pm$2.0%
10μ	10μL	$\pm$1.5%	$\pm$1.5%
50μ	1μL	$\pm$5.0%	$\pm$5.0%
50μ	5μL	$\pm$2.5%	$\pm$2.0%
50μ	50μL	$\pm$2.0%	$\pm$1.0%
300μ	10μL	$\pm$5.0%	$\pm$2.0%
300μ	50μL	$\pm$2.0%	$\pm$1.0%
300μ	300μL	$\pm$1.0%	$\pm$1.0%
1000μ	10μL	$\pm$7.5%	$\pm$3.5%
1000μ	100μL	$\pm$2.0%	$\pm$1.0%
1000μ	1000μL	$\pm$1.0%	$\pm$1.0%

Table 1.2: **NIMBUS4 Pipette Specifications [17]**

The price of the Hamilton NIMBUS4 was quoted for a minimum of $\$35,000$ CA without the inclusion of the gripper, extra pipettors, or accessories, though it can be found on the used market for around $\$14,786$ CAD.

1.2.3 Beckman Coulter Biomek 4000

Figure 1.5: **Beckman Coulter Biomek 4000 [18].**

The Biomek 4000 is an automated liquid handler developed by Beckman Coulter Life Sciences. It features 12 deck slots with an off-deck add-on slot that supports the addition of four additional labware, a thermocycler, a shaker, or a waste disposal unit. Most standard labware such as wellplates, tubestands, reservoirs, etc. are compatible with the Biomek deck. The Biomek 4000 supports pipettors capable of moving volumes between 1μL and 1000μL. A gripper is included for moving labware between deck positions. The Biomek hardware and software are not open-source.

There are a total of six different pipettors available for purchase: three single-channel pipettors and three 8-channel pipettors. One major advantage of the Biomek is the lack of required proprietary pipette tips. It is possible to use tips that are already in use by a lab. Beckman Coulter does not provide accuracy or precision for the pipettors likely due to multi-compatibility of tips. The accuracy may change drastically between pipette tips.

The Biomek 4000 is no longer for sale, but used devices can be found for around $\$20,000$ CAD. The newest replacement was quoted for around $\$150,000$ USD.

1.2.4 OpenTrons OT-2

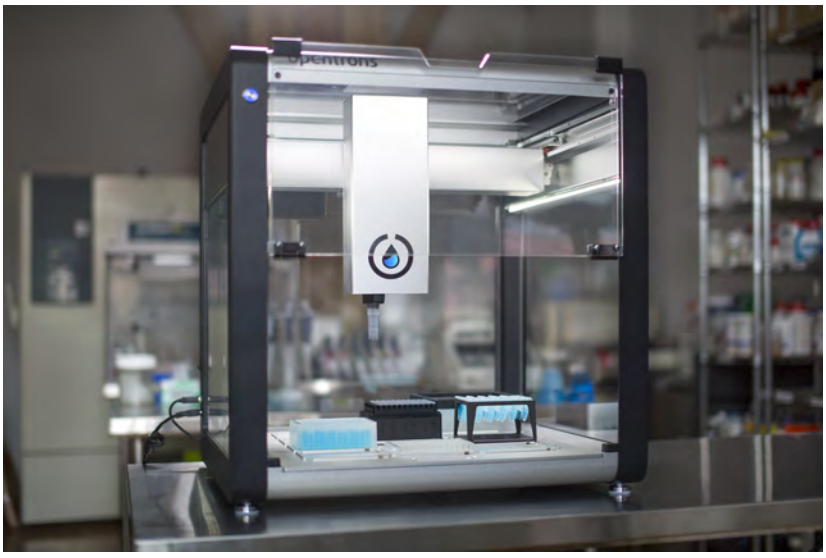


Figure 1.6: **OpenTrons-OT2 with 8-channel pipette [19].**

The Opentrons OT-2 system is the highest performing commercially available liquid handler in terms of features, versatility, and performance in the category of sub $\$10,000$ USD liquid handlers. The bare-bones

system starts at $\$6,000$ USD with the option of 5 exchangeable pipettes. There are 3 single channel options including a P20, a P300, and a P1000 each costing $\$1,250$ USD. There are also 2 eight-channel pipettes supporting P20 and P300 each costing $\$2,000$ USD. The P20, P300, etc. refer to the type of pipette tip that the pipette supports. Images of the various supported tips can be seen in figure 1.7. The OT-2 platform supports up to 11 different combinations of labware including, but not limited to tip boxes, tubestands, 96-wellplates, and reservoirs. A removable trash bin is included with the OT-2. While Opentrons sells its own officially supported labware, the OT-2 can accommodate labware that the end-user already has available. Opentrons also sells several add-on modules including a heater shaker module, a thermocycler module, a temperature module, a magnetic module, and a HEPA air filter module for removing particulate and contaminates [20].

(a) (b)

Figure 1.7: **(a) OpenTrons OT-2 single channel pipettes [21]. (b) OpenTrons OT-2 eight channel pipettes [22].**

The specifications for the single-channel pipettes and the 8-channel pipettes can be seen below in table 1.3 and table 1.4 respectively.

Pipette	Volume	Accuracy %D	Accuracy μL	Precision %CV	Precision μL
P20 Gen2	1	$\pm$15%	0.15μL	$\pm$5%	0.05μL
P20 Gen2	10	$\pm$2%	0.2μL	$\pm$1%	0.1μL
P20 Gen2	20	$\pm$1.5%	0.3μL	$\pm$0.8%	0.16μL
P300 Gen2	20	$\pm$4%	0.8μL	$\pm$2.5%	0.5μL
P300 Gen2	150	$\pm$1%	1.5μL	$\pm$0.4%	0.6μL
P300 Gen2	300	$\pm$0.6%	1.8μL	$\pm$0.3%	0.9μL
P1000 Gen2	100	$\pm$2%	2.0μL	$\pm$1%	1μL
P1000 Gen2	500	$\pm$1%	5.0μL	$\pm$0.2%	1μL
P1000 Gen2	1000	$\pm$0.7%	7.0μL	$\pm$0.15%	1.5μL

Table 1.3: **OpenTrons-OT2 single-channel pipette specifications [23]**

Pipette	Volume	Accuracy %D	Accuracy μL	Precision %CV	Precision μL
P20 Gen2	1	$\pm$20%	0.2μL	$\pm$10%	0.1μL
P20 Gen2	10	$\pm$3%	0.3μL	$\pm$2%	0.2μL
P20 Gen2	20	$\pm$2.2%	0.44μL	$\pm$1.6%	0.3μL
P300 Gen2	20	$\pm$10%	2.0μL	$\pm$4%	0.8μL
P300 Gen2	150	$\pm$2.5%	3.75μL	$\pm$0.8%	1.2μL
P300 Gen2	300	$\pm$1.5%	4.5μL	$\pm$0.5%	1.5μL

Table 1.4: **OpenTrons-OT2 8-channel pipette specifications [23].**

For both the single-channel and 8-channel pipettes, OpenTrons uses a linear actuator system consisting of a stepper motor, lead screw, and coupler that attaches the stepper motor to piston as seen in figure 1.8. Each pump uses a limit switch for resetting the volume piston position. In addition, each pipette supports tip ejection.

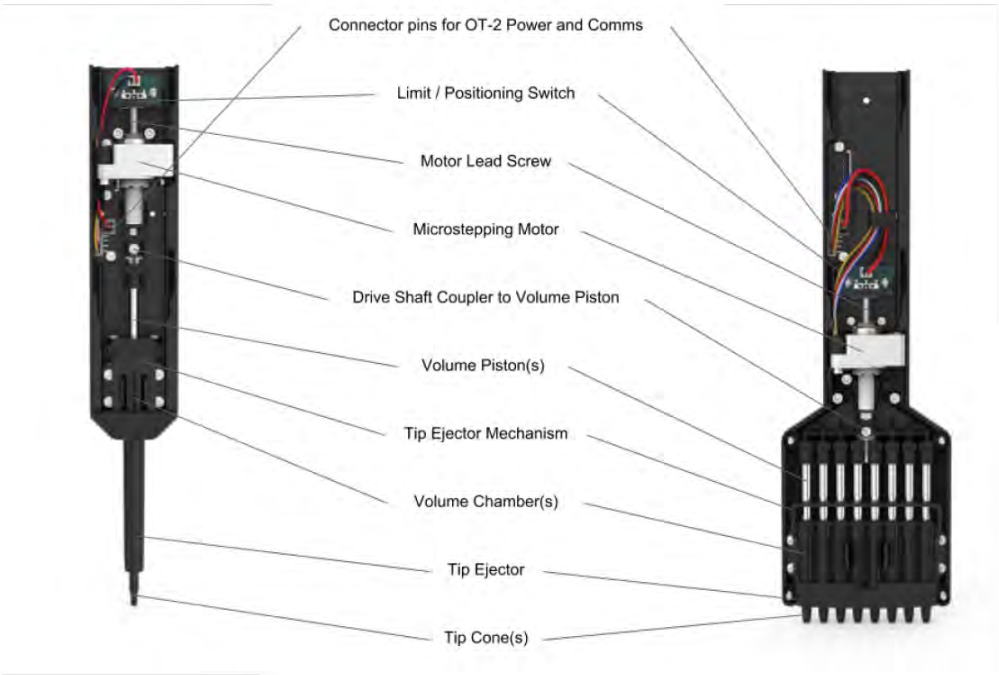


Figure 1.8: **OpenTrons-OT2 pipette design diagram [24].**

The OT-2 is controlled via programs written in Python. Each protocol is designed to interface with the custom OT-2 Python Protocol API. Each protocol consists of metadata such as the protocol name, contact information, etc., the labware, and how the OT-2 should behave [25]. The protocols are designed to be easy to read and easy to program for those that have basic knowledge of Python and wet-lab work. A sample protocol can be seen in pseudo-code listed below in Listing 1.1.

Listing 1.1: **Opentrons OT-2 sample protocol code [25].**

```
from opentrons import protocol_api

# metadata
metadata = {
    'protocolName': 'My Protocol',
    'author': 'Name <opentrons@example.com>',
    'description': 'Simple protocol to get started using the OT-2',
    'apiLevel': '2.13'
```

```
}


# protocol run function
def run(protocol: protocol_api.ProtocolContext):


    # labware
    plate = protocol.load_labware('corning_96_wellplate_360ul_flat',
        location='1')
    tiprack = protocol.load_labware('opentrons_96_tiprack_300ul',
        location='2')


    # pipettes
    left_pipette = protocol.load_instrument(
         'p300_single', mount='left', tip_racks=[tiprack])


    # commands
    left_pipette.pick_up_tip()
    left_pipette.aspirate(100, plate['A1'])
    left_pipette.dispense(100, plate['B2'])
    left_pipette.drop_tip()
```

While OpenTrons makes the designs open-source, they are

1.2.5 EvoBot

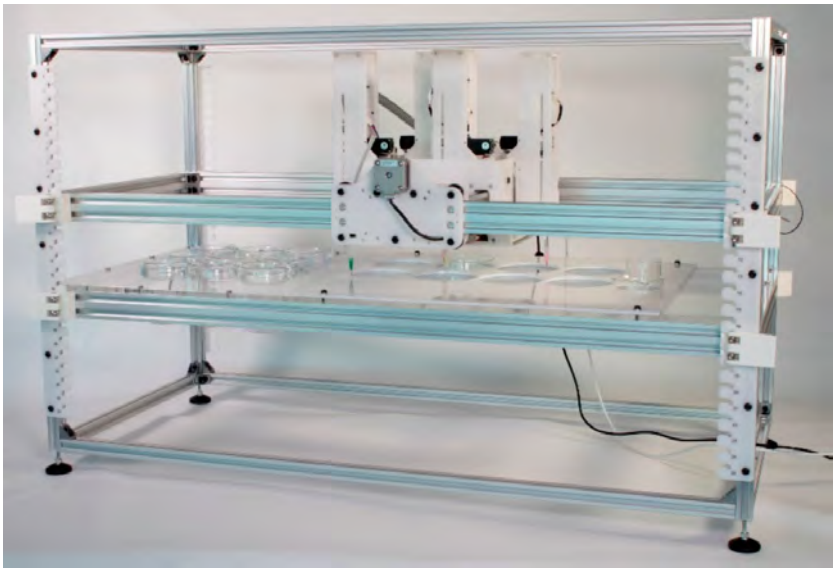


Figure 1.9: **EvotBot liquid handler [26].**

The EvoBot, originally developed by Faiña et al in 2016 [27], improved by Nejatimoharrami, Faiña, and Stoy in 2017 [28] and improved again by Faiña, Nejati, and Stoy in 2019 [26], is a modular, open-source liquid handling bot for automating synthetic biology experiments. With its three kinds of modules, it is a versatile device capable of automating basic liquid handling, nurturing of microbial fuel cells, droptlet chemotaxis and more [26]. The latest iteration of the EvoBot is designed primarily with off-the-shelf 3D printer components, laser cut, and 3D printer parts.

The frame of the EvoBot is 600mm x 400 mm x 600 mm, but is designed to be extendable if large metal extrusions are used. The EvoBot is designed with three layers: an actuation layer, an experiment layer, and an observation layer [26]. The design of the Evobot can be seen below in figure 1.10 and modules can be seen in figure 1.11b. The actuation layer supports various modules for different functions. There are four different syringe modules supporting up to 100μL, 1mL, 5mL, and 20mL respectively. The minimum supported volumes are not specified. The dispensing module is designed for pumping up to four liquids and is used for tasks such as washing Petri dishes or dispensing reagents. The heavy payload can safely move heavy loads and is used to hold a 3D scanner and a universal paste extruder.

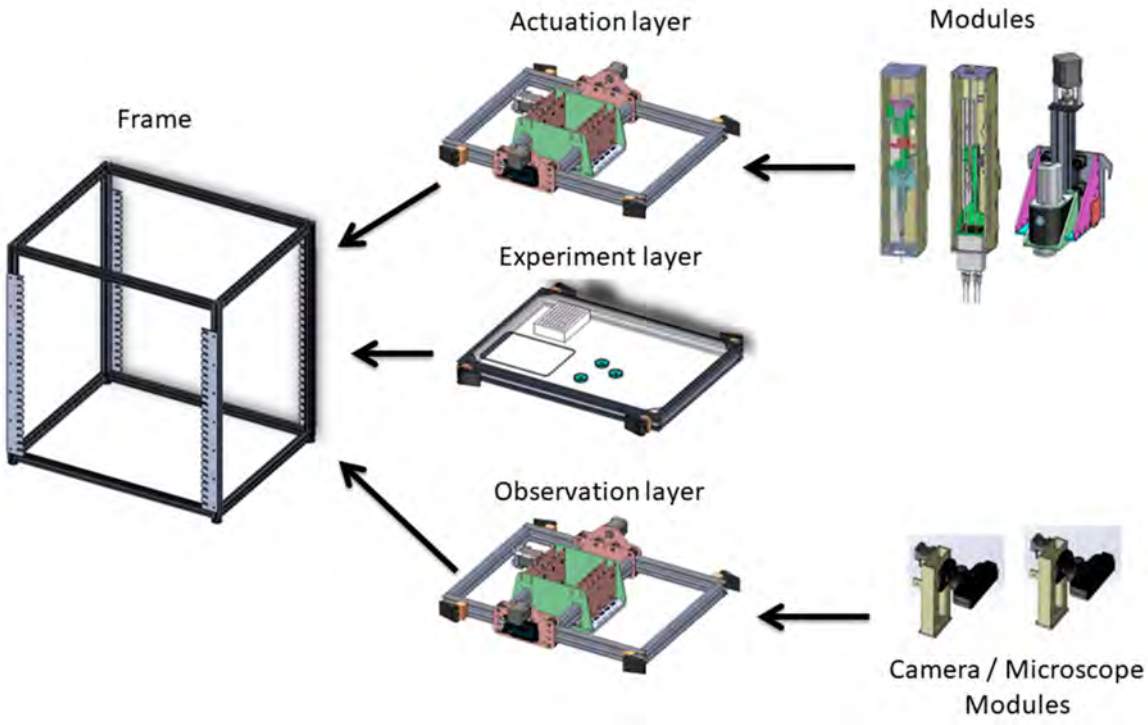
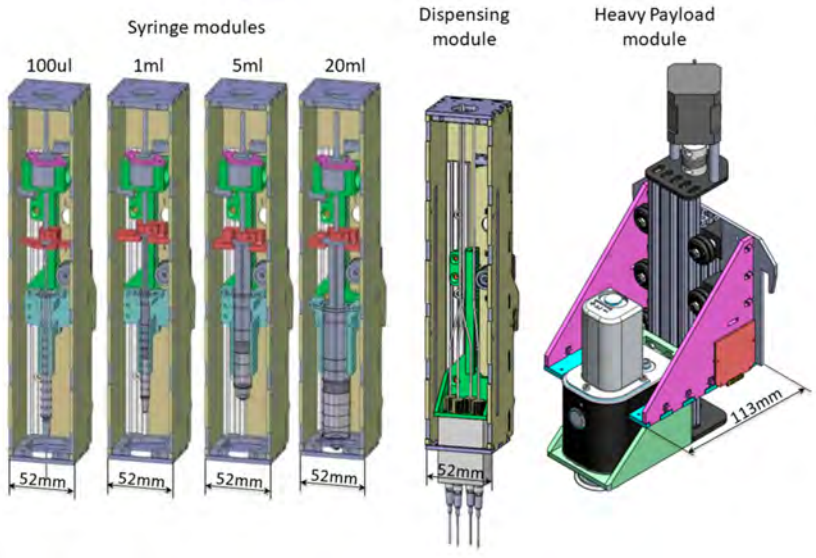
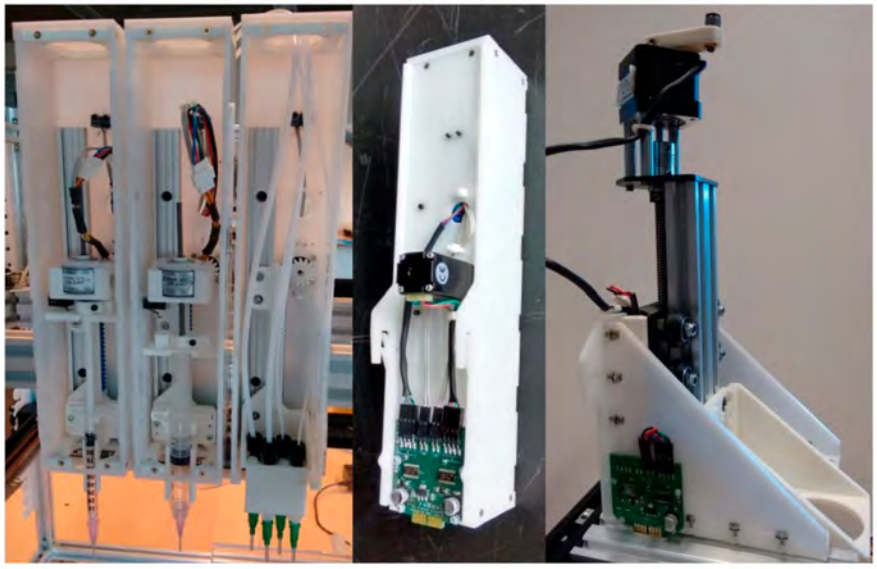


Figure 1.10: **Overview of EvoBot modularity. The various layers and modules can be swapped depending on the requirements of an experiment [26].**



(a)



(b)

Figure 1.11: **(a) Drawings of the syringe modules, dispensing module, and heavy payload module. (b) Image of the actual modules. [26].**

The syringe modules are designed using a NEMA 8 stepper motor that rotates a gear to actuate the syringe along a linear rail. Each of the four syringe modules follow an identical design using slightly modified 3D printed parts for holding the compatible syringe. Each syringe module includes two limit switches for tracking the volumes moved in the syringe. The general design of the syringe modules and the design of the heavy module can be seen in figure 1.12. Each module uses a standardized custom-PCB for controlling the actuators and sensors.

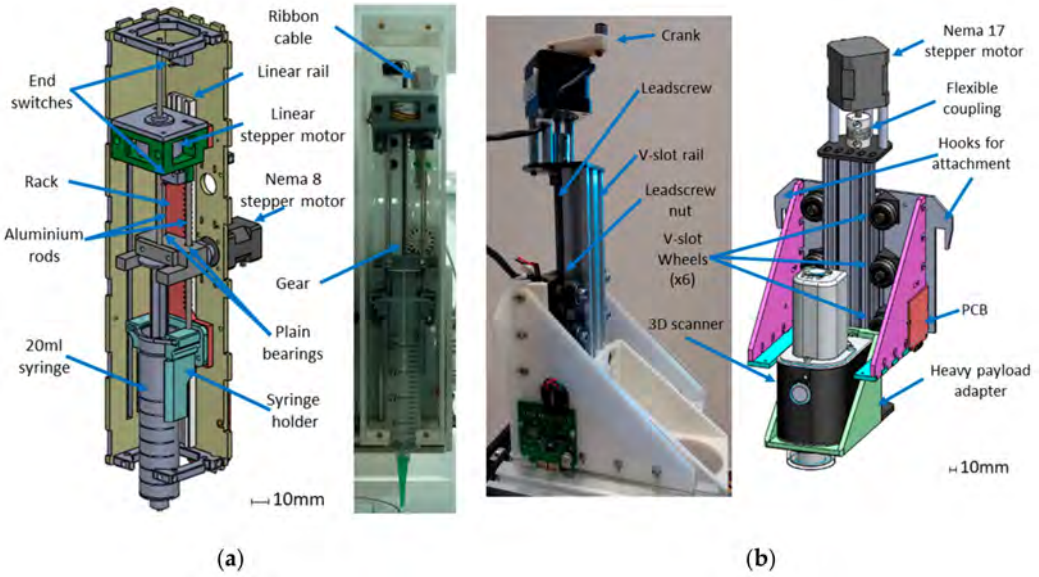
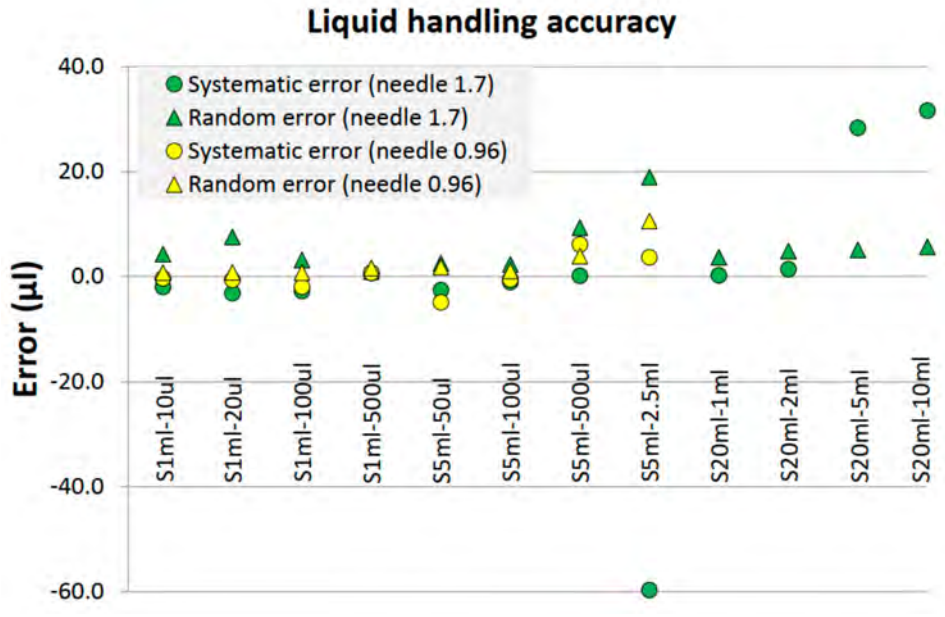
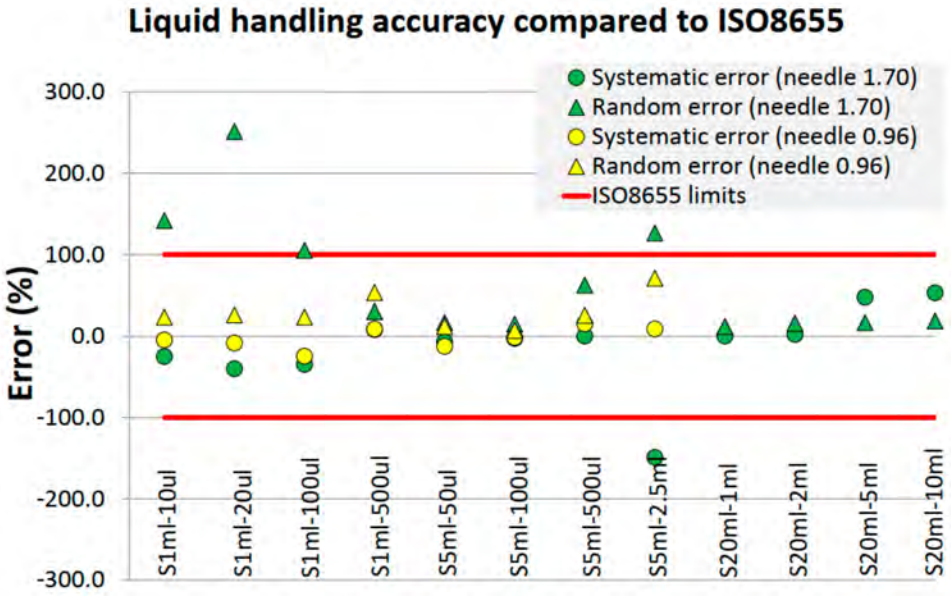


Figure 1.12: **(a) Design of syringe module. (b) Design of heavy payload module. [26].**

The syringe modules were designed to meet the ISO8655 standards which can be seen at [29] and [30]. Faiña et al verified functionality using non-contact gravimetrical tests, meaning the syringe needle does not touch the liquid when dispensing. The results of the tests are seen below in figure 1.13.



(a)



(b)

Figure 1.13: **(a) EvoBot syringe modules accuracies. (b) EvoBot syringe modules accuracies with ISO8655 standard for comparison. [26]**

According to the authors, the standard Evobot configuration with one actuation layer, one experimental layer, and a single syringe module costs approximately $600 USD.

1.2.6 OTTO

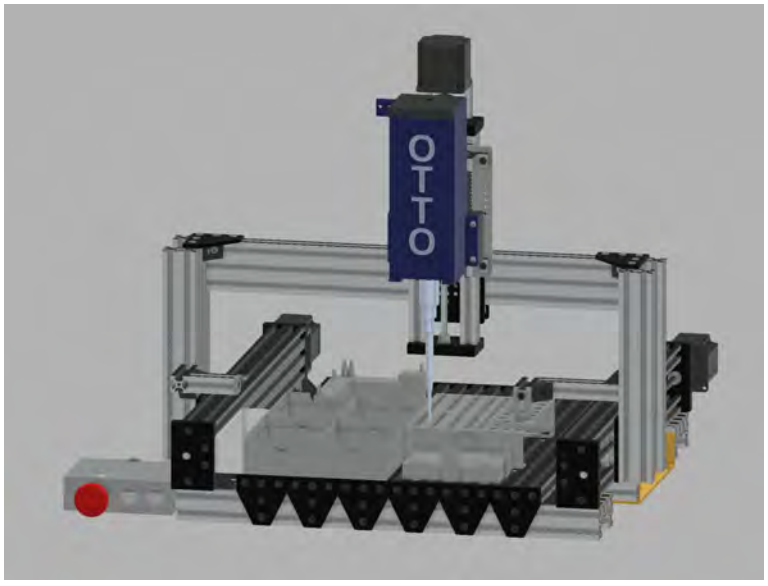


Figure 1.14: **Figure of OTTO liquid handler from openliquidhandler website [31].**

OTTO is an open-source automated liquid handler designed for automating qPCR [32]. It features off-the-shelf and 3D printed components. It consists of a pump carriage (which uses a manual pipette), a 3-axis movement system consisting of linear actuators, and a two-beam laser system for tip attachment/removal verification. The frame is constructed using easily sourced metal extrusions and supports up to 4 pipette tip boxes, 8 medium sized Eppendorf tubes, 64 PCR tubes, and a 96 PCR wellplate. Each motor is controlled via an Arduino Due, which features a 32-bit ARM microprocessor, and TMC2660 motor drivers.

The syringe pump utilizes a linear actuator to control a manual pipette. Once the pipette volume is set by the user, the pump aspirates and dispenses by actuating the pipette according to the desired volume. The pump uses two springs to attach pipette tips with constant pressure and detect z-axis collisions to prevent damaging the pipette.

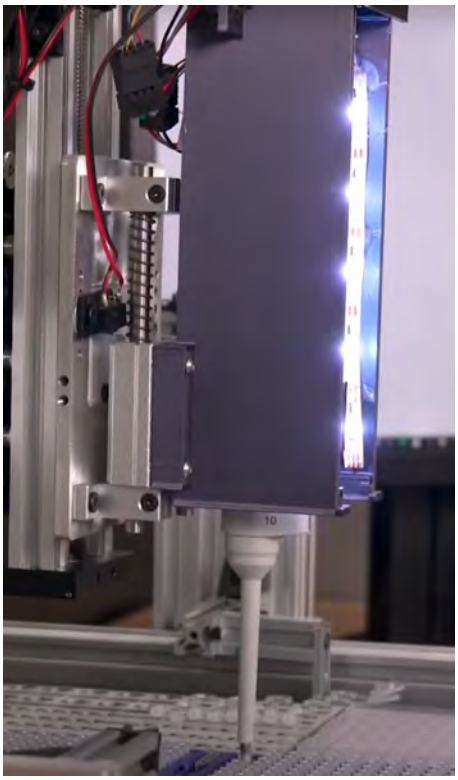


Figure 1.15: **Side view of OTTO pipettor. The springs assist in pipette tip attachment and help detect collisions through the use of a limit switch [33].**

OTTO includes open-source software for the user to design custom qPCR experiments. While the software is currently limited to qPCR with a 96-wellplate, it includes placeholders for the addition of dilution experiments, flow experiments, 384-wellplates, and flow tubes. According to the bill of materials provided

by Florian et al., OTTO costs upwards of $\$2,000$ USD [34].

1.3 Summary

The following tables summarize the key characteristics of each of the previously explored devices.

Device	**Creator**	**Supported Volume Range**	**Minimum Cost (CAD)**	**Open-Source Software**	**Open-Source Hardware**
epMotion 5075t	Eppendorf	1μL-1,000μL	$\$110,000$	No	No
Microlab NIMBUS4	Hamilton	1μL-1,000μL	$\$35,000$	No	No
Biomek 4000	Beckman Coulter	1μL-1,000μL	$\$20,000$ used	No	No
OpenTrons-OT2	OpenTrons	1μL-1,000μL	$\$8,736$	Apps and protocols only	Designs only
EvoBot	Faiña et al. [26]	10μL-10,000μL	$\$806$	Yes	Yes
OTTO	Florian et al. [32]	NA	$\$2,015$ \$	Yes	Yes

Table 1.5: **Comparison of liquid handler devices.**

Device	**Add-Ons**
epMotion 5075	Multiple pipettors, ThermoMixer (included), gripper (included)
Microlab NIMBUS4	Multiple Pipettors, labware gripper, barcode scanner, shft-n-scan tube barcode scanner, CO-RE paddles, $[MPE]^2$ positive pressure solid phase extraction and evaporation module, CO-RE lid tool, vacuum station, clean air system, heater shaker, plate tilt module, fill module
Biomek 4000	Multiple pipettors, ALP labware stand, tube rack ALP, auto-latch tip rack holders, orbital shaker, disposal accessory, liquid waste station, bar code reader, circulating reservoir, filtration system, off-deck platform, thermal exchange unit, wash system
OpenTrons-OT2	Multiple pipettors, thermocycler, temperature module, magnetic module, HEPA module, heater-shaker module
EvoBot	Multiple pipettors, dispensing module, heavy payload module

OTTO	None, but could accommodate

Table 1.6: **Table of automated liquid handlers' add-ons.**

Device	**Volume**	**Accuracy**	**Precision**
epMotion 5075	1μL	$\pm$20.0%	$\pm$5.0%
epMotion 5075	5μL	$\pm$5.0%	$\pm$3.0%
epMotion 5075	25μL	$\pm$1.5%	$\pm$0.6%
epMotion 5075	50μL	$\pm$1.0%	$\pm$0.4%
epMotion 5075	100μL	$\pm$2.0%	$\pm$1.0%
epMotion 5075	200μL	$\pm$0.6%	$\pm$0.3%
Microlab NIMBUS4	1μL	$\pm$5.0%	$\pm$5.0%
Microlab NIMBUS4	5μL	$\pm$2.5%	$\pm$2.0%
Microlab NIMBUS4	25μL	NA	NA
Microlab NIMBUS4	50μL	$\pm$2.0%	$\pm$1.0%
Microlab NIMBUS4	100μL	$\pm$2.0%	$\pm$1.0%
Microlab NIMBUS4	200μL	$\pm$1.0%	$\pm$1.0%
Biomek 4000	NA	NA	NA
OpenTrons-OT2	1μL	$\pm$20.0%	$\pm$5.0%
OpenTrons-OT2	5μL	NA	NA
OpenTrons-OT2	20μL	$\pm$1.5%	$\pm$0.8%
OpenTrons-OT2	50μL	NA	NA
OpenTrons-OT2	100μL	$\pm$2.0%	$\pm$1.0%
OpenTrons-OT2	300μL	$\pm$0.6%	$\pm$0.3%
EvoBot	NA	NA	NA
OTTO	NA	NA	NA

Table 1.7: **Comparison automated liquid handlers' pipettor accuracy and precision.**

Chapter 2

Requirements & Specifications

This chapter outlines the user requirements and technical specifications of the BioCloneBot. These requirements are used to make the design decisions for each component of the device. Table 2.1 provides a high-level summary of the device requirements. Note that some of these requirements are implicitly extracted from the requirements outlined in the following sections.

ID	Category	Requirement
1.1	General	The device shall consist of off-the shelf components and 3D printed parts.
1.2	General	The device shall perform operations such as attaching pipette tips, removing pipette tips, aspirating volumes, dispensing volumes, and mixing volumes.
1.3	General	Each 3D printed part shall fit the 225mm x 225mm bed of an Ender 3 Pro.
2.1	Syringe Pump	The pump shall move volumes between 1.0μL and 200.0μL
2.2	Syringe Pump	The pump shall be designed to eject pipette tips.
3.1	Platform	The labware platform dimensions shall be 400mm x 400mm.
3.2	Platform	The platform shall support standard labware including wellplates, tubestands, and tip boxes.
3.3	Platform	The platform shall support up to 6 individual labware or sub-components.
4.1	Electrical	The power supply shall have a 3.3V, 5.0V, and 12.0V rail.
4.2	Electrical	The power supply shall supply a minimum of 15A on the 12.0V rail.
5.1	Firmware	The firmware should be easily expandable.
5.2	Firmware	The firmware should only perform simple calculations.
5.3	Firmware	The firmware loop should be as simple as possible.

6.1	Front-End	The front-end shall support adding, removing, and modifying labware volumes or tip availability.
6.2	Front-End	The front-end shall provide a user interface to support the operations defined in requirement 1.2.
6.3	Front-End	The front-end shall support saving and loading experiments including labware, initial conditions, and performed operations.
7.1	Biology	The device shall be able to perform the liquid handling steps of Restriction Enzyme Digestion and Ligation.
7.2	Biology	The device shall be able to perform the liquid handling steps of partial Gibson Assembly.
7.3	Biology	The device shall be able to perform the liquid handling steps of Golden Gate Cloning.

Table 2.1: **High-Level Device Requirements**

2.1 User Requirements

The ultimate goal of the BioCloneBot is to provide a versatile liquid handler capable of automating the liquid handling steps of the Restriction Enzyme Digestion and Ligation, Gibson Assembly, and Golden Gate Cloning protocols. To realize the necessary steps, the device and corresponding software must support the ability to automatically attach its pipette tips. Once a tip is attached, it should also support the ability to aspirate a volume and then dispense said volume into the target labware. Most experiments are sensitive to contamination, and as such the device must also be able to eject the attached tip. To make the device more accessible, it needs to consist of off-the-shelf components and 3D-printed parts. Since the primary stakeholders are biologists, the device should be easy to assemble, program, and use. The software should be simple while maintaining sufficient flexibility to support most liquid handling protocols and already available labware. The software should include the ability to save and load the protocols defined in the software to help realize the time-saving benefits of automation.

2.2 Technical Specifications

Before the BioCloneBot can be designed, assembled, and tested, the technical specifications must be defined. To lower the cost of construction, all 3D printed parts must fit the 225mm x 225mm bed of the Ender 3 Pro as it is one of the cheapest quality 3D printers currently available. The method for moving volumes must be able to accurately aspirate and dispense volumes between 1-20μL as the three protocols work on volumes in this range. While the optimal accuracy target is to meet the ISO 8655 standard for manual pipettes, most commercial liquid handlers operate with higher levels of error. As seen in the literature review, most devices do not support 1μL, but if they do, they operate with an error of 15-20%. The BioCloneBot should meet or reduce the 20% error threshold. For volumes larger than 1μL, most of the reviewed device errors range between 0.5-5.0%. Again, the BioCloneBot should meet this standard. While the three chosen protocols use less than 20μL of liquid, the device should be able to move volumes up to 200μL to make the device versatile enough to support a large range of liquid handling applications. Since most electronics work on 3.3, 5.0, or 12.0V, the BioCloneBot must support these voltages to allow for future expandability of devices such as a thermocycler which allows for complete automation up to the end of the PCR steps.

In summary, the goal is to create an easy-to-construct, program, and operate device capable of moving volumes between 1μL and 200μL for the purpose of automating the liquid handling steps of the Restriction Enzyme Digestion and Ligation, Gibson Assembly, and Golden Gate Cloning protocols. The device must also be designed with expandability and versatility in mind. All of these requirements lead to the following chapter where the device is designed and constructed.

Chapter 3

Materials, Construction, & Operation

This chapter describes the design decisions for creating the BioCloneBot such that user requirements and technical specifications are met. A complete assembly guide, software guide, and bill of materials can be found in A, B, and C, respectively. To realize the BioCloneBot, five major components were chosen: a microliter syringe pump to perform pipette actions, a 3-axis movement system and device frame for moving the syringe pump, a power system for powering all the electronics, a microcontroller for controlling motors and related electronics, and an intuitive front-end for designing experiments. The requirements can be found in table 2.1 from Chapter 2.

3.1 Microliter Syringe Pump

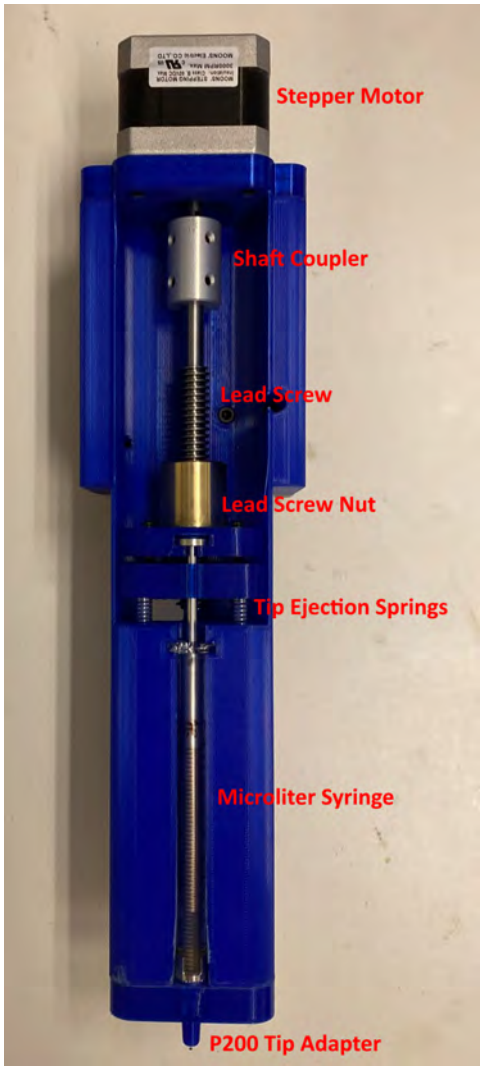


Figure 3.1: **Custom Microliter Syringe Pump with P200 Tip Adapter.**

Since most of the reviewed devices use a linear actuated pump, a syringe pump design was chosen. Inspired by the designs of the OpenTrons pump, the EvoBot syringe modules, and the re-usable DIY syringe pumps from Amarante et al. [35] and Gervasi, Cardol, and Meyer [36], a custom microliter syringe pump was designed. Stepper motor based syringe pumps are advantageous as they are highly precise and easy to control. Notably, stepper motor based lead screw or pulley systems are used for applications such as 3D printing indicating a proper system for the purpose of an accurate, low volume syringe pump. The chosen pump design consists of several major parts: a microliter syringe, a linear actuator consisting of a 12.0

V, 1.5 A 400 step bipolar stepper motor, a 2 mm pitch lead screw, a 5 mm to 6 mm shaft coupler, a lead screw nut, and 3D printed components. A limit switch and two springs were included for added pipette tip removal functionality. The lead screw was chosen due to its small pitch and short length. Finer pitched lead screws can be used, but are drastically more expensive or require machining to be cut to size violating requirement 3.4. The pump supports either a 50μL syringe for volumes between 1.0-20.0μL or a 250μL syringe for volumes between 10.0-200.0μL to satisfy requirement 3.1. The 3D printed parts are compatible with other 700 series Hamilton syringes with little to no modifications, but for the purpose of this thesis only the 50μL and 250μL syringes will be tested. A 3D printed P20 tip adapter and P200 tip were designed to accommodate both types of pipette tips. To ensure the syringe has an airtight seal with the tip adapter, each tip adapter was glued to to the syringe RN nut using a two part epoxy. In addition, the syringe must be pre-filled with dH_2O to ensure the syringe plunger has a good seal. If the syringe is run dry, leakage can occur resulting in failed aspiration or dispensing. According to the Hamilton website, the 50μL syringe has an inner diameter of 1.03mm and the 250 μL syringe has an inner diameter of 2.30mm [16]. The maximum travel distance of both syringes is 60mm. With this information, the minimum and maximum volumes can be calculated. By coupling a 400-step stepper motor with a 2mm pitch lead screw, the result is a highly accurate and highly precise linear actuator. A full revolution of the stepper motor (400 steps) causes the lead screw nut to travel the 2mm pitch of the lead screw. The minimum linear distance traveled by 1 step is calculated as follows

$$D = \frac{1}{s_{rev}} \times p \tag{1}$$

where s_{rev} is the number of steps per revolution of the motor, and p is the pitch of the lead screw in mm.

$$D = \frac{1}{400} \times 2 = 0.005 \frac{mm}{step} \tag{2}$$

With the minimum travel distance of 0.005mm/step, the theoretical minimum aspiration volume of the 50μL can then be calculated to be

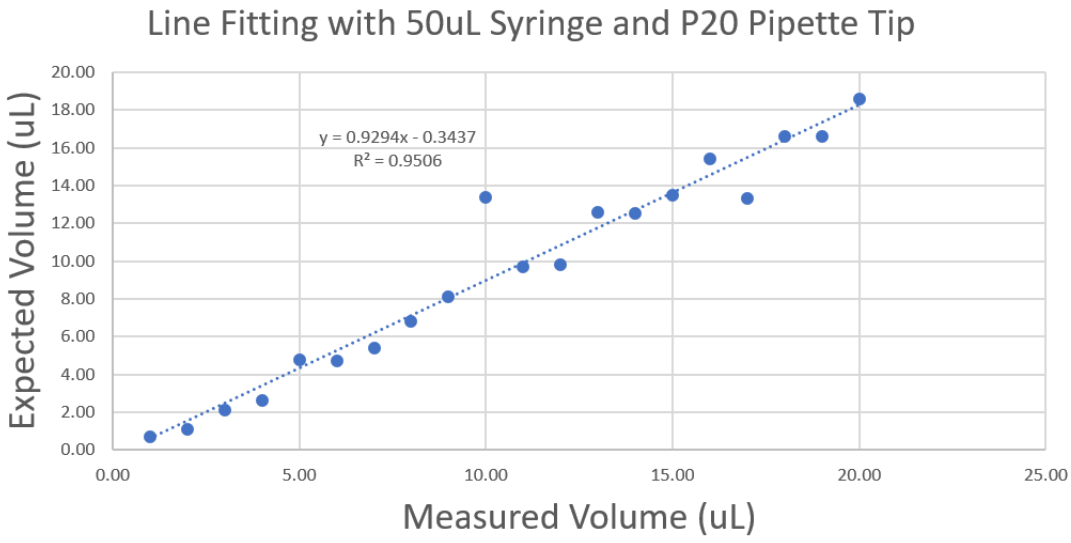
$$V_{min} = \frac{0.005}{60} \times 50 = 0.00417 \mu L \tag{3}$$

The linear actuator was designed with a maximum travel distance of $\approx 50mm$. When using the 250μL syringe, the upper limit is calculated to be

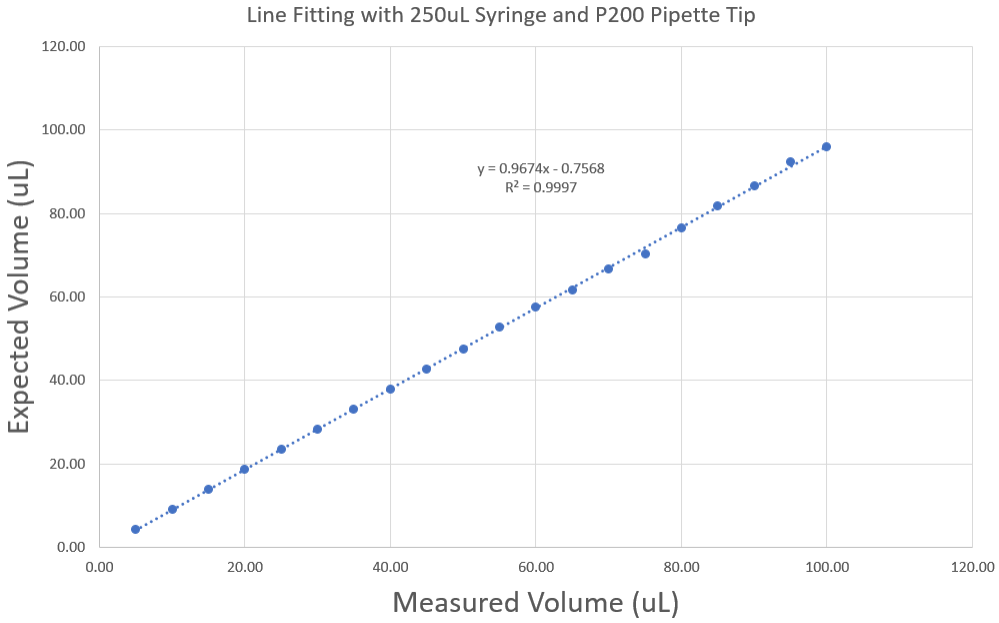
$$V_{max} = \frac{50}{60} \times 250 = 208.33\mu L \tag{4}$$

With a theoretical minimum of 0.00417μL using and a maximum of 208.33μL, requirement 3.1 is satisfied. To satisfy requirement 3.2, the pump was designed using springs and a limit switch for automatic tip removal. When the lead screw nut reaches the bottom of the pump, the springs are compressed and the limit switch is pressed resulting in any attached tip being removed. By pressing the limit switch, the pump is homed removing all errors introduced by hysteresis which is a build-up of the historical error over time.

During initial testing, each aspirated volume was found to be short of the target volume by a consistent quantity. To help offset this systemic error, a range of volumes was measured, the measured volumes were plotted against the target volumes, and a line was fit to the data. This process was performed for the 50μL syringe using the P20 tip adapter with a P20 tip and the 250μL syringe using the P200 tip adapter with a P200 tip. The 50μL syringe was tested using volumes from 1μL to 20μL in 1μL increments and the 250μL syringe was tested using volumes from 5μL to 100μL in 5μL increments. The resultant line equations can be found in figure 3.2 below. This correction lowered the average error substantially for both syringes.



(a)



(b)

Figure 3.2: **The high R^2 value indicates that both lines fit the data with a high correlation. (a) Graph of measured volumes vs target volumes using P20 tip adapter and P20 tips. (b) Graph of measured volumes vs target volumes using P200 tip adapter and P200 tips.**

The final design consisted of a microliter syringe pump capable of moving small volumes as low as 1μL required for the key three protocols and large volumes up to 200μL to accommodate a wide range of liquid handling protocols. The design was created in such a way that the pump is homed each time a pipette tip is ejected.

3.2 3-Axis Movement System & Device Frame

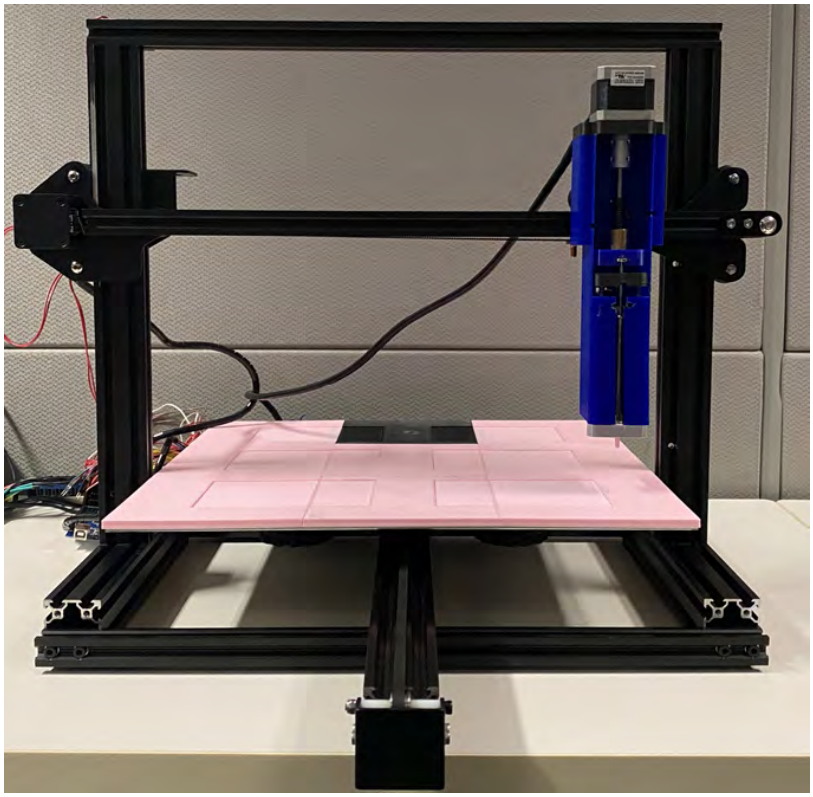
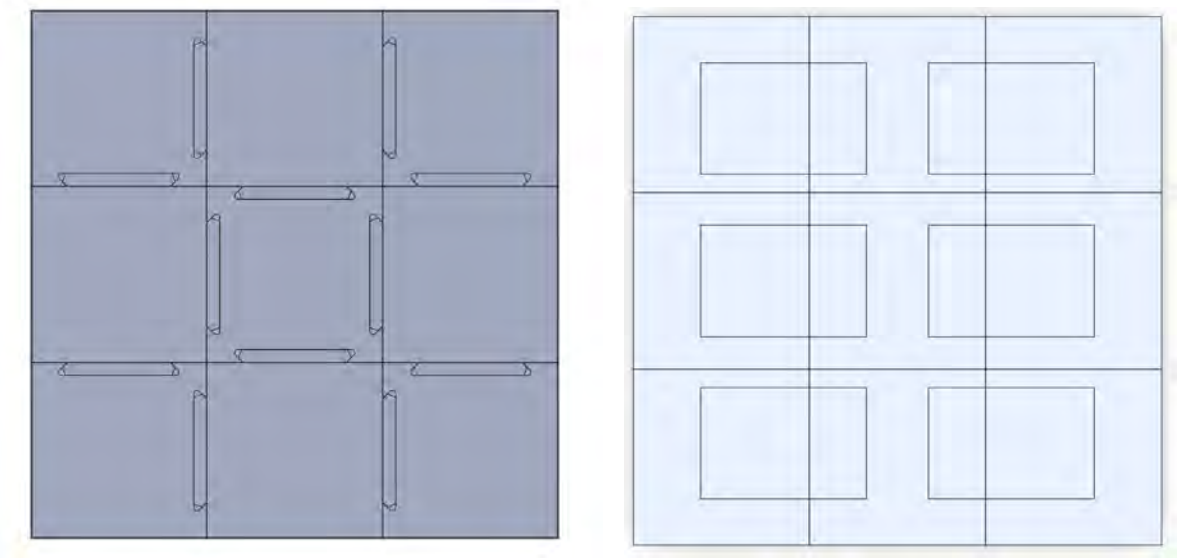


Figure 3.3: **BioCloneBot frame and pump fully assembled.**

Following a similar design to the OTTO and Eva liquid handlers, an Ender 3 Pro 3D printer was chosen to provide the basis of a mechanical frame and platform to hold labware. An Ender Xtender 400 kit was included to increase the size of the bed from 225mm x 225mm to 400mm x 400mm for partial satisfaction of requirement 2.3. The Ender 3 Pro and Ender Xtender 400 kit is $200-$300 cheaper than the next largest printer, the Creality Ender 5 Plus, which comes with a smaller bed dimension of 350mm x 350mm. This combo makes up the entire frame of the BioCloneBot and includes an already existing x, y, and z system with stepper motors and limit switches proven to work efficiently with highly precise movements required for 3D printing. With the larger bed size, the platform supports up to 6 labware. Given the maximum print width of 225mm by the Ender 3 Pro, the platform is designed as 9 individual parts that connect together with dove-tail fittings as seen in figure 3.4 below. Each labware slot is 128mm x 86mm to support the standard dimension of a wellplate. Additional 3D printed adapters can be designed to fit support different sized labware such as tubestands and tipboxes.



(a) (b)

Figure 3.4: **(a) Bottom of platform with dove-tail fittings. (b) Top-down view of the platform with the 6 labware slots.**

To control movement of the syringe pump, the original stepper motors of the Ender are used. While the Ender 3 Pro mainboard could be used for controlling the motors, it offers no GPIO ports for expandability. As such, an Arduino Mega 2560 and four DRV8825 stepper motor drivers were selected to control the x, y, z, and pump stepper motors. The DRV8825 supports microstepping as low as 1/32 which provides substantially higher precision. The minimum theoretical travel distance is calculated using the linear travel distance of a tooth pulley belt system for the x and y axes

$$D = \frac{p \times N_t}{s_{rev} \times f_m} \tag{5}$$

where p is the pitch of the belt in mm, N_t is the number of pulley teeth, s_{rev} is the number of steps per revolution of the motor, and f_m is the microstepping factor of the motor.

$$D_{x\_min} = \frac{2 \times 20}{200 \times 32} = 0.00625mm \tag{6}$$

$$D_{y\_min} = \frac{2 \times 20}{200 \times 32} = 0.00625mm \tag{7}$$

The minimum distance of the z-axis requires an extra calculation compared to the syringe pump. The Ender z-axis lead screw has a pitch of 2mm and a lead of 4 which means when the lead screw nut makes a full rotation, a total of four pitches will be traveled or 8mm. The lead of a screw refers to how many individual threads are on the screw. A screw that has a single lead has one thread and a screw with four leads has four separate threads wrapped around the screw. z_{min} can be calculated using the following equation

$$D = \frac{1}{s_{rev}} \times L \times f_{rm} \tag{8}$$

where L is the pitch times the lead.

$$D_{z\_min} = \frac{1}{200} \times 8 \times \frac{1}{32} = 0.00125mm \tag{9}$$

D_{x_min}, D_{y_min}, and D_{z_min} satisfy requirement 3.4 given that the distance between wells of a 96 wellplate and tips in a 96 tip box is 9mm.

3.3 Power System

The BioCloneBot uses a 450 watt computer PSU due its high current rating at 12.0V. The Ender PSU is rated for 15A at 24V which provides sufficient power, but requires additional circuitry to step down to 12.0V, 5.5V, and 3.3V. Unlike the Ender PSU, a computer power supply includes 3.3V and 5.0V lines allowing for the addition of most consumer grade electronics such as sensors and fans. Initially the BioCloneBot was designed to support the addition of a custom built thermocycler which required approximately 15.0A at 12.0V. Due to time limitations, this was not included in the final prototype of the BioCloneBot. A wiring diagram of the system can be found in Appendix A.

3.4 Microcontroller

While the Ender 3-Pro uses the open source 3D printer firmware, Marlin, the code base is large and has an even larger learning curve to understand and modify [37]. To reduce the complexity of the firmware code and ensure ease of modification for future improvements, the entire BioCloneBot firmware is implemented

from scratch. An Arduino Mega 2560 was chosen due to the large quantity of GPIO ports allowing individual control for each of the four DRV8825 drivers. The firmware was designed to use a custom made communication protocol. Each message is built as a string with the start character '#' and the end character '%'. The first four characters of each message are encode a 4-bit binary number. This allows for up to 16 different commands. The characters following the 4-bit encoding dictates the parameters of the command. A list of the commands is described in **Table 3.1** below. 9 commands are implemented allowing for up to 7 additional commands.

Command	4-bits	Parameters	Description
Home	0000	None	Homes x, y, z, and pump stepper motors.
Move	0001	XYZ direction XXX.XX mm YYY.YY mm ZZZ.ZZ mm	Move pump in XYZ direction, XXX.XX mm, YYY.YY mm, and ZZZ.ZZ mm
Remove Tip	0010	None	Moves pump over trash bin, removes tip, and homes pump.
Aspirate	0011	XXX.XX μL	Aspirates XXX.XX volume into pipette tip.
Dispense	0100	XXX.XX μL	Dispenses XXX.XX volume into pipette tip.
Mix	0101	XYZ times XXX.XX volume	Mixes XXX.XX volume XYZ amount of times.
Enable Steppers	1110	None	Enables all stepper motor drivers.
Disable Steppers	1111	None	Disables all stepper motor drivers.

Table 3.1: **List of Device Commands**

As an example, a set commands is described below.

(1) #0000%

(2) #0001100100.00000.00000.00%

(3) #0011100.00%

First, #0000% triggers the BioCloneBot to move to the (0, 0, 0), identical to the origin of a standard Cartesian coordinate system. Note that 0 on the z-axis refers to the highest point instead of the lowest point.

Once in the (0, 0, 0) position, the pump actuates until the limit switch is engaged, backs-off until the limit switch is no longer engaged, and then backs-off a little further to create a trailing air-gap. This helps remove the error introduced by backlash created by the lead screw/lead screw nut.

Second, the command, #0001100100.00000.00000.00%, moves the pump to the position (100.00, 0, 0). The first four characters after the start character #, 0001, indicates the move command. The following three characters, 100, indicates the directions the pump will move in each axis. 0 means towards the limit switch and 1 means move away from the limit switch. The 1 means moves away from the limit switch in the x-axis and following 00 means move towards the limit switch in the y-axis and z-axis respectively. The last portion can be broken up into three groups of 6 characters: XXX.XX, YYY.YY, and ZZZ.ZZ. 100.00 indicates that the pump should move 100.00 mm in the x-axis. The last 000.00 000.00 indicates that the pump should not move in either the y-axis or the z-axis.

Last, #0011100.00% tells the pump to aspirate 100.00μL. The first four characters, 0011, indicates the aspirate command and the last 6 characters, 100.00, indicates that the pump should aspirate by 100.00μL. Thus ending the commands.

The firmware consists of the pin definition/global variable definition, the setup function, and the loop function. The following code snippits provide high-level pseudo-code of the firmware. For exact code, see the GitHub repository linked in C . The pin definition indicates which Arduino ports are used and the declares the global variables such as the number of steps/revolution of the motors, travel distances, and the command messages. The setup function is described using the follow pseudo-code.

Listing 3.1: **Firmware setup function pseudo-code**

```
Define pin modes as INPUT, OUTPUT, or INPUT_PULLUP
Set initial LOW or HIGH value of pins
Set delay duration between steps
Set pump steps per revolution
Set pump travel distance per step
Set X, Y, and Z axis microstepping (1/32 by default)
Initialize LCD screen
Initialize serial connection with front-end
```

Overall, the setup function is simple. Once all the pins are defined and initialized, the serial connection between the microcontroller and the front-end is established through an exchange of specific messages. The front-end initializes the handshake with a simple message and once the Arduino has received the message, responds indicating that the serial port connection is successful.

The loop function was designed to be as simple and light weight as possible. The majority of calculations are performed by the substantially more powerful front-end. The workflow of the loop function is described in the pseudo-code below.

Listing 3.2: **Firmware loop function pseudo-code**

```
Reset distances, volumes, and mix count
Listen on serial port for start character "#"
    If "#" is received
        Read serial input until end character "%" is received
        Decode 4-bit command encoding
        Jump to if statement for decoded command
        Process command parameters if exist
        Home, move pump, eject tip, aspirate, dispense, or mix
        Send completion message over serial to front-end
```

As seen, the firmware is designed to follow a short and simple workflow satisfying requirement 5.3.

3.5 Front-End

Last, the BioCloneBot requires a front-end to allow the end user to design their own experiments. C# Sharp was chosen due to the easy-to-use graphical user interface design capabilities of Visual Studio. This limits the BioCloneBot to Windows devices which should be available in most lab settings. Within the front-end, the user must design experiments by first adding labware, and then initializing labware properties such as starting volumes, starting tip locations, etc. Once the labware has been decided, the user can generate an experiment by selecting operations. A user guide is available in Appendix B.

The front-end is designed to be simple and easy to use. Partially inspired by the user interfaces of the Biomek and the OpenTrons, the user interface is designed with the majority of the functionality built directly

into the main window as seen in figure 3.5 below.

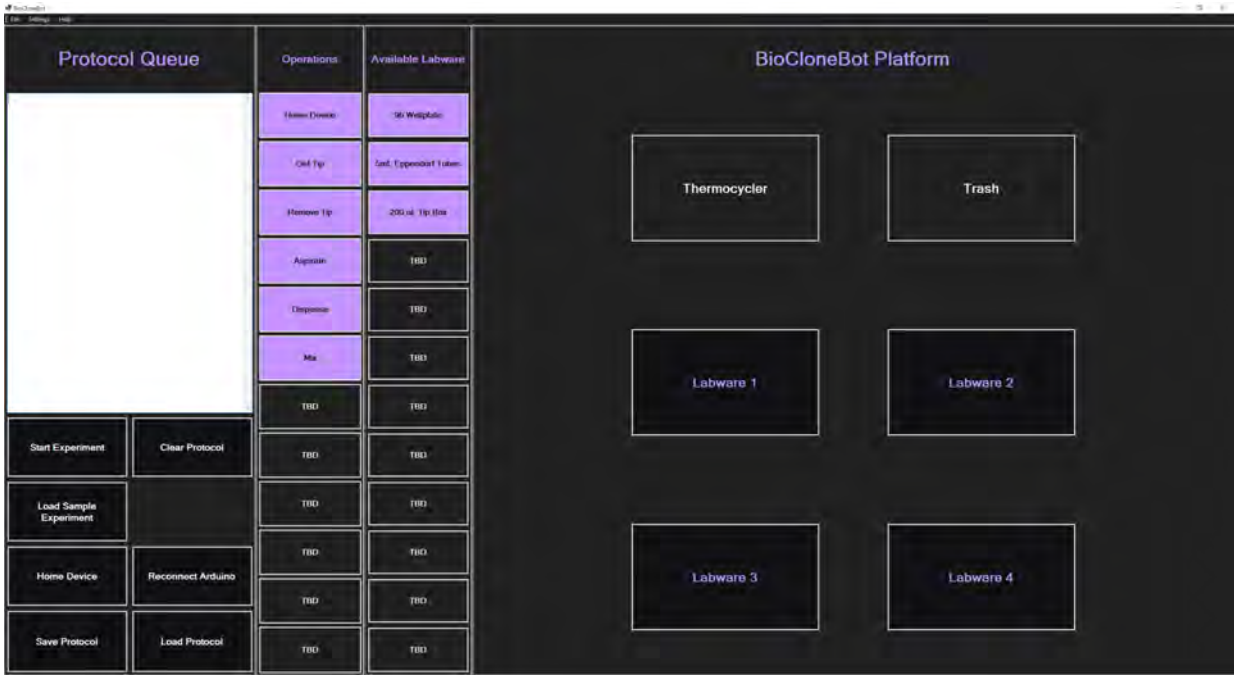
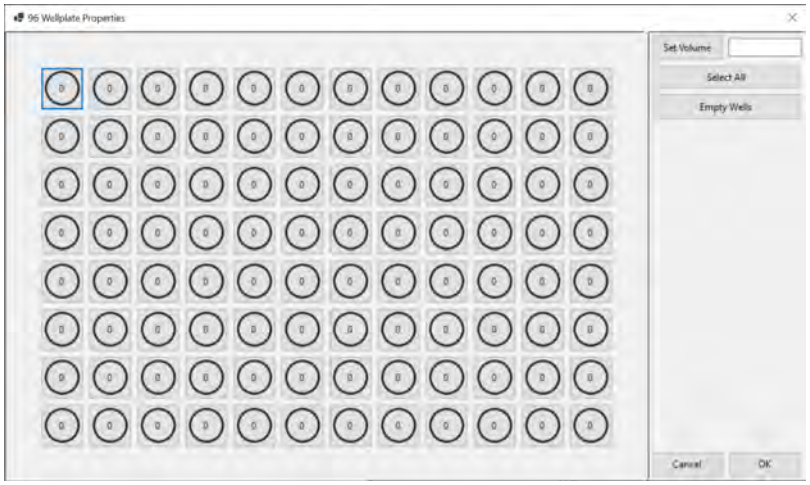


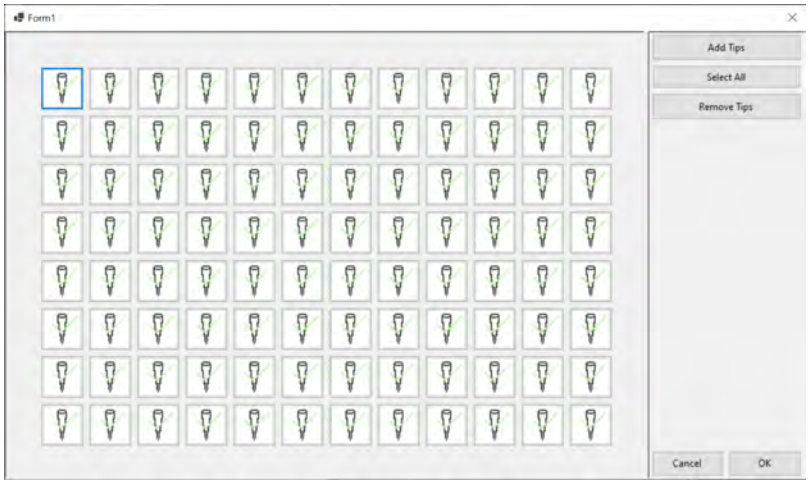
Figure 3.5: **BioCloneBot Front-End**

The physical platform is displayed on the right side of the figure. Here any of the Available Labware can be added, removed, and modified. To the left of the platform, there is a column for available labware. By default, only a standard 96-wellplate, a 5mL Eppendorf tubestand consisting of a 4x6 configuration capable of holding 5mL Eppendorf tubes, and a 200μL tipbox is available. Once a labware is added, the properties can be modified by double clicking on the labware corresponding labware slot. The labware properties for each of the standard labware can be seen below in Figure A.1.


96 Wellplate Properties
Set Volume
Select All
Empty Wells
Cancel
OK

(a)

(b)



(c)

Figure 3.6: **(a) 96-wellplate properties window. (b) 5mL Eppendorf tubestand properties window. (c) 200μL tipbox properties window. The properties window supports the selection of individual wells, tubes, or tips. The select all button allows for quick modification of reservoir volumes or tip availability. Modifications are only confirmed once pressing the OK button.**

The operations column includes buttons to home the device, get a new tip, remove the current tip, aspirate a volume, dispense a volume, and mix a volume satisfying requirements 1.2 and 6.2. When adding an operation to the protocol, the selected operation displays underneath the Protocol Queue. In addition, the software includes several buttons which are described in Table 3.2 below. The ability to save and load experiments satisfies requirement 6.3. The software is designed such that it performs checks verifying that conditions are met before performing an operation. For instance, the Get Tip operation requires that an available tipbox labware exists on the platform.

Button	Description
Start Experiment	Starts the designed experiment.
Clear Protocol	Removes all added labware and operations.
Load Sample Experiment	Loads a basic sample experiment.
Home Device	Homes the device identical to the Home Device operation
Reconnect Arduino	Attempts to reconnect to the Arduino. Useful after updating the firmware.
Save Protocol	Saves all added labware, labware properties, and operations to a JSON file.
Load Protocol	Loads all added labware, labware properties, and operations from a saved JSON file.

Table 3.2: **Front-end Control Buttons.**

For troubleshooting and calibration, the Manual Control Debug window can be used. Each operation from the main BioCloneBot window can be performed individually. The Manual Control Debug window can be seen in figure 3.7 below. The window allows a specified distance to be moved in either direction on a specified axis. For example, if 10 is entered and the +x button is pressed, the pump will move 10.00mm away from the limit switch on the x-axis.

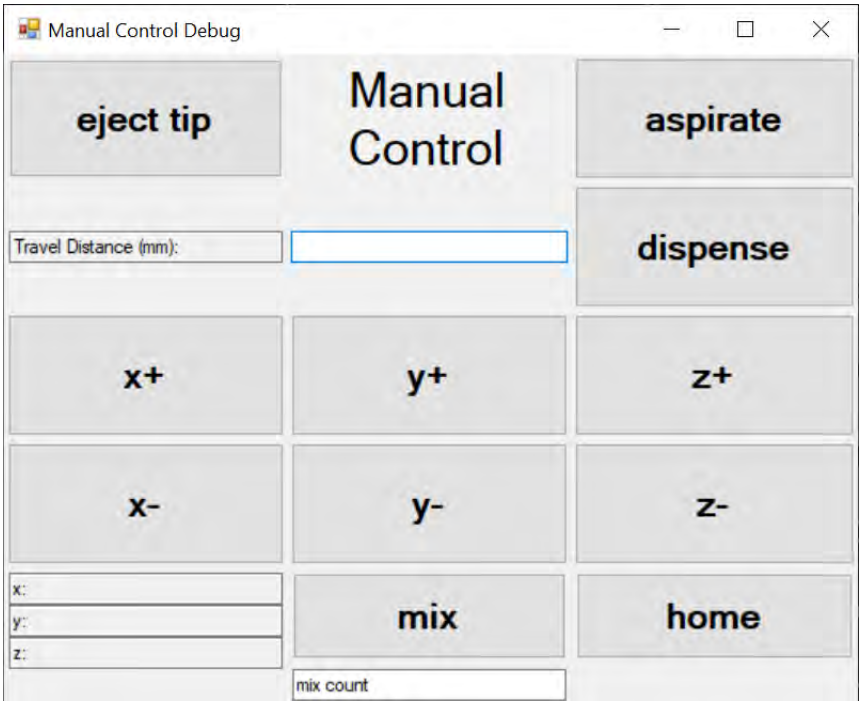


Figure 3.7: **Manual Control Window for calibration and troubleshooting.**

3.6 Summary

In summary, the BioCloneBot was designed using an Ender 3 Pro and Ender Xtender kit to provide a cheap frame with stepper motors and limit switches included. This frame provides a stepper motor powered 3-axis system for moving a custom built microliter syringe pump. The syringe pump supports either a 50μL or 250μL microliter syringe from Hamilton. To aspirate volumes, dispense volumes, and eject tips, a stepper motor powered linear actuator system was designed. The pump includes a limit switch for detecting when a tip is ejected and for homing the pump. To control the 3-axis and syringe pump stepper motors, an Arduino Mega 2560 and four DRV8825 stepper motor drivers were selected. The firmware was programmed using a custom communication protocol that consists of a 4-bit encoding which dictates what operation the device should perform (move pump, aspirate, dispense, etc.). The rest of the messages consists of the parameters of the operation. The front-end, which was programmed in C#, was designed with to generate user-defined protocols with to perform liquid handling operations with 96-wellplates, tubestands, and tipboxes.

Chapter 4

Results

This chapter is divided into two sections. The first section details the tests and results from validating the performance of the BioCloneBot device. The second section provides the biological methods and results of automating the liqquid handling portion of the chosen protocols with the BioCloneBot: Restriction Enzyme Digestion & Ligation, Gibson Assembly, and Golden Gate Cloning.

4.1 Device Characterization

4.1.1 Syringe Pump Volume Characterization

To validate and characterize the syringe pump, a procedure was developed for measuring the volume of each complete aspiration and dispensing. Each volume was found by using a Sartorius CP64 scale which is accurate down to 0.1mg. The volumes were tested in two batches. The first batch consisted of volumes between 1μL and 20μL using the P20 tip adapter and the second batch consisted of volumes between 10μL and 200μL using the P200 tip adapter. The testing procedure is as follows (n=10 for each volume)

(1) Remove syringe from syringe pump.

(2) Fill the syringe with dH20. This creates a perfect seal around the syringe plunger.

(3) Place the syringe back into the syringe pump.

(4) Open BioCloneBot software and run homing procedure (some water will exit the syringe, this is OK).

(5) Manually attach pipette tip.

(6) Aspirate trailing air of 5μL for the 50μL syringe or 10μL for the 250μL syringe.

(7) Weigh empty tube.

(8) Using a tube full dH_2O, aspirate the adjusted test volume according to the line fit equation from Chapter 3.

(9) Dispense adjusted volume + $5mu$L for the 50μL syringe or 10μL for the 250μL syringe into the weighed tube.

(10) Weigh tube filled with water.

(11) Calculate volume of water using this equation: (weight of tube with dH_2O - weight of empty tube)/0.001 to convert from grams of dH_2O to μL of dH_2O.

(12) Repeat the process 10 times for each test volume.

The results for each test volume are seen in table 4.1 below. The equations used for calculating the accuracy and the precision are as follows

Calculate the mean of the measured volumes for a specific target volume

$$\bar{V} = \frac{1}{n} \times \sum_{i=1}^{n} V_i \tag{10}$$

Next, calculate the accuracy in percentage where V_s is the target volume

$$e_s = \frac{100(\bar{V} - V_s)}{V_s} \tag{11}$$

Calculate the repeatability standard deviation

$$S_r = \sqrt{\frac{\sum_{i=1}^{n}(V_i - \bar{V})^2}{n-1}} \tag{12}$$

Next, calculate the precision

$$CV = 100 \times \frac{S_r}{\bar{V}} \tag{13}$$

Syringe/Pipette Tip	Target Volume (μL)	Adjusted Volume (μL)	Accuracy (%)	Precision (%)
50μL P20	1.00	1.45	$\pm$5.00%	$\pm$11.22%
50μL P20	2.00	2.52	$\pm$12.50%	$\pm$7.03%
50μL P20	5.00	5.13	$\pm$2.60%	$\pm$6.03%
50μL P20	10.00	11.13	$\pm$0.80%	$\pm$5.17%
50μL P20	20.00	21.89	$\pm$1.05%	$\pm$0.97%
250μL P200	10.00	11.12	$\pm$2.60%	$\pm$1.47%
250μL P200	25.00	26.62	$\pm$0.32%	$\pm$0.39%
250μL P200	50.00	52.47	$\pm$0.74%	$\pm$0.36%
250μL P200	100.00	104.15	$\pm$0.93%	$\pm$4.80%
250μL P200	200.00	207.52	$\pm$0.95%	$\pm$0.32%

Table 4.1: **Results of syringe pump tests using the adjusted volumes derived from the line fitting from section 3.2 of chapter 3. Each volume was tested 10 times. The largest error occurs with the smaller volumes and progressively gets lower as the volume increases. This is also observed with all of the reviewed syringe pumps.**

Volumes lower than 1μL were tested, but a wide range of errors were observed resulting in a lower limit of 1μL to maintain accuracy. The adjusted volume in 4.1 refers to volume the pump tries to aspirate to actually aspirate a desired volume. For example if a 1μL aspiration is desired, the pump is told to aspirate 1.45μL. This results in the aspiration much closer to 1μL. If the pump was told to aspirate 1μL, then the real volume would be closer to 0.8μL. The equations for the adjusted volumes can be found in 3.2 from Chapter 3. To minimize the effects of air compression, backlash, and left over volumes, a leading air volume was aspirated before aspirating dH_2O and a trailing air volume is dispensed after dispensing dH_2O. Each test was performed by weighing an empty Eppendorf tube, aspirating the specified volume of dH_2O into a fresh pipette tip, dispensing the specified volume into the same Eppendorf tube, then weighing the tube with the added volume. Since dH_2O was used, it is assumed that 1μL of dH_2O is equivalent to 1 gram. The final volume is calculated by subtracting the weight of the empty tube from the weight of the dH_2O + tube, then converting the result from grams to μL.

The same testing procedure was performed to test the syringe pump with mixtures of varying viscosity. To calculate the final volumes of each mixture, the following densities were used: 1.031941 g/cm^3 for 12.5%

glycerol, 1.054393 g/cm^3 for 25% glycerol, 1.083871 g/cm^3 for 37.5% glycerol, and 1.115044 g/cm^3 for 50% glycerol. All of the following tests were performed using the 50μL syringe, the P20 tip adapter, a P20 pipette tip, and the P20 adjusted volumes.

Glycerol Percentage	**Target Volume (μL)**	**Adjusted Volume (μL)**	**Accuracy (%)**	**Precision (%)**
0%	1.00	1.45	±5.00%	±11.22%
0%	3.00	3.60	±9.00%	±6.12%
0%	5.00	5.75	±5.0%	±11.22%
0%	7.00	7.90	±7.14%	±3.00%
12.5%	1.00	1.45	±22.37%	±15.66%
12.5%	3.00	3.60	±20.48%	±15.17%
12.5%	5.00	5.75	±16.48%	±6.44%
12.5%	7.00	7.90	±12.27%	±5.71%
25%	1.00	1.45	±71.66%	±28.11%
25%	3.00	3.60	±15.39%	±8.09%
25%	5.00	5.75	±13.51%	±8.54%
25%	7.00	7.90	±18.91%	±24.18%
37.5%	1.00	1.45	±31.01%	±14.77%
37.5%	3.00	3.60	±19.64%	±16.89%
37.5%	5.00	5.75	±11.23%	±10.68%
37.5%	7.00	7.90	±9.13%	±2.60%
50%	1.00	1.45	±75.78%	±26.80%
50%	3.00	3.60	±27.35%	±12.77%
50%	5.00	5.75	±11.92%	±6.89%
50%	7.00	7.90	±11.21%	±4.60%

Table 4.2: **Results of testing glycerol and dH$_2$O mixtures at 0% glycerol, 12.5% glycerol, 25% glycerol, 37.5% glycerol, and 50% glycerol. Each volume was tested 10 times. Despite the different viscosities, the error is approximately the same for each mixture.**

4.1.2 Syringe Pump Tip Attachment/Removal Characterization

To validate the ability of the BioCloneBot to attach and remove tips, a simple test was performed. The setup for the test is as follows:

(1) Calibrate BioCloneBot for 20μL pipette tip box.

(2) Load tip box with 96 tips.

(3) In BioCloneBot software, add a Tip Box labware to labware slot 1.

(4) Add Home Device operation.

(5) Add Get Tip operation.

(6) Add Remove Tip operation.

(7) Repeat Get Tip and Remove Tip operations for half of the tip box.

(8) Add Home Device operation (to remove any hysteresis-induced error).

(9) Add Get Tip operation.

(10) Add Remove Tip operation.

(11) Repeat Get Tip and Remove Tip operations for remainder of the tip box.

(12) Run experiment.

After approximately 1.5 hour of running, all 96 tips were successfully attached and then removed. While this does not guarantee that each tip was attached with a perfect seal, it clearly demonstrates the repeatability of the BioCloneBot to attach and remove pipette tips. Given that the 96-tip box matches the dimensions of a 96-wellplate, it can be extrapolated that the device is capable of targeting a 96-wellplate with sufficient accuracy. The speed of the BioCloneBot has a drawback in terms of speed. The OTTO liquid handler is capable of preparing an entire 96-wellplate qPCR reaction in less than an hour. The OpenTrons is capable of a similar 96-wellplate experiment requiring the transfer of volumes from one 96-wellplate to another 96-wellplate while changing tips in between transfers in a little over 35 minutes.

4.1.3 X, Y, and Z Stepper Motor Characterization

By analyzing the footage of a performed experiment using VLC Media Player, rough velocities of the system were calculated. The x-axis was found to have a velocity of 50.74 mm/s while traveling 202.95mm, the y-axis was found to have a velocity of 48.11 mm/s while traveling 250.55mm, and the z-axis was found to have a velocity of 8.23 mm/s while traveling 74.1mm. These measurements were made by calculating the travel distance over a period of time according to the video. Frame-by-frame analysis was done to find exact seconds when the pump starts and stops moving for each axis.

From the calculations, the maximum travel distance for each axis was calculated: X-Axis: 400mm/50.74mm/s = 8 seconds Y-Axis: 400mm/48.11mm/s = 8 seconds Z-Axis: 135mm/8.23mm/s = 16 seconds

The accuracy and precision of the x, y, and z stepper motors were approximated using calipers. The manual control form was used to make 10mm movements. After each movement, a line was drawn using a pen and the distance between the lines was measured. This measurement was performed n=10 times for each axis. The results can be seen in the table 4.3 below.

Axis	Test Distance (mm)	Accuracy (%)	Precision (%)
X-Axis	10.0	$\pm 0.15\%$	$\pm 0.98\%$
Y-Axis	10.0	$\pm 0.70\%$	$\pm 1.14\%$
Z-Axis	10.0	$\pm 0.53\%$	$\pm 1.37\%$

Table 4.3: **Results of x, y, and z stepper motor tests. n=10 movements of 10mm was performed for each axis. A line was drawn at the destination and measured from the previous line using calipers.**

4.2 Verification of Functionality

After characterizing the functionality of the syringe pump, the overall functionality of the BioCloneBot was tested. Restriction Enzyme Digest And Ligation, Golden Gate Assembly, and Gibson Assembly were all run and compared to a manual manipulation via a pipette.

4.2.1 Restriction Enzyme Digest

A restriction enzyme digest and ligation of a GFP-dropout insert was performed. Using a single plasmid containing GFP, a digestion was performed to remove the GFP flanking two BsaI cut sites. After digestion, the original GFP was religated back into the backbone. Using the tubestand labware, a total of three tubes were used. The pump picked up volumes ranging between 3μL to 11μL and placed them into a collection tube total 19μL. At the time of this test, only a 500μL syringe was available and as such, was used to perform the protocol. The P200 tip adapter was used with P200 pipette tips. In between each aspiration and dispensing step, the pipette tip was removed into the trash bin then replaced with a clean tip. Once each input was combined together, the resulting collection tube volume was moved to a PCR tube, then run through a thermocycler. The resulting DNA plasmid was transformed into E. coli cells and then plated. After plating, a mini-prep was run and the resulting DNA was run through a gel electrophoresis for final confirmation. The process for automating the liquid handling portion of the experiment in the BioCloneBot software is outlined below:

(1) Add tubestand labware to platform.

(2) Add tipbox labware to platform.

(3) Set initial condition of tube 1 to 15μL.

(4) Set initial condition of tube 2 to 10μL.

(5) Set initial condition of tube 3 to 11μL.

(6) Add get tip operation.

(7) Add aspirate operation aspirating 6μL from tube 1.

(8) Add dispense operation dispensing 6μL into tube 3.

(9) Add remove tip operation.

(10) Add get tip operation.

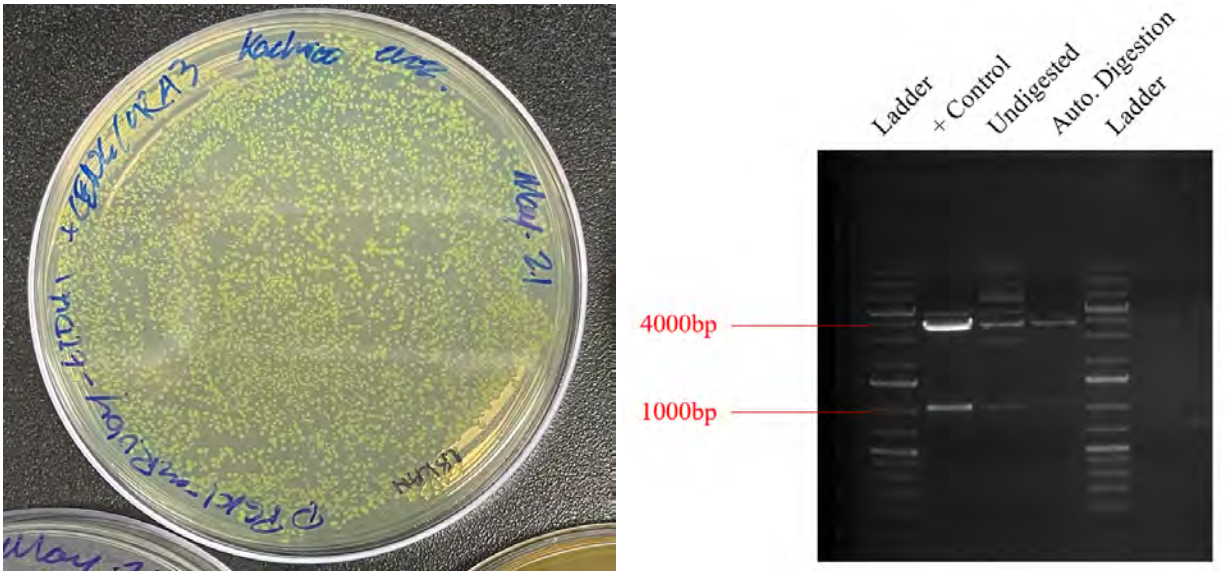
(11) Add aspirate operation aspirating 3μL from tube 2.

(12) Add dispense operation dispensing 3μL into tube 3.

(13) Add mix operation mixing 10μL 5 times on tube 3.

(14) Add remove tip operation.

(15) BioCloneBot step is complete. Transfer contents of tube 3 into PCR tube and run amplification.



(a) (b)

Figure 4.1: **Restriction enzyme digest of CEN6/URA3 Kan+ plasmid with GFP dropout insert. Digestion with restriction enzyme BsaI results in a separation of the backbone at 3721bp and the GFP insert at 1026bp. The full plasmid length is 4747bp. (a) Transformation of E. coli cells after ligation of backbone to GFP. Green color indicates the fluorescence of GFP as the normal color is brownish. Plated on LB and Kanamycin.(b) Gel electrophoresis of manually digested CEN6/URA3 and automated digestion of CEN6/URA3 via the BioCloneBot with 1kb+ ladder. Positive control indicates a band below 4kb and a band at 1kb.**

4.2.2 Gibson Assembly

A Gibson Assembly was performed to insert a GFP insert into a linearized pCEN6/URA3 Kan+ backbone. The protocol was performed using three wells of a standard 96-wellplate labware. The pump picked up volumes ranging between 1μL to 3μL and placed them into a collection well totaling 19.39μL. The 50μL syringe was used with the P20 tip adapter and P20 pipette tips. In between each aspiration and dispensing step, the pipette tip was removed into the trash bin then replaced with a clean tip. Once each input was combined together, the result was manually moved to a single PCR tube, then run through a thermocycler. The resulting DNA plasmid was transformed into E. coli cells and then plated. After plating, a mini-prep was run and the resulting DNA was run through a gel electrophoresis for initial confirmation. The final DNA was sent for sequencing and returned with 99.98% sequence match. The process for automating the liquid handling portion of the experiment in the BioCloneBot software is outlined below:

(1) Add 96 wellplate labware to platform.

(2) Add tipbox labware to platform.

(3) Set initial condition of wellplate reservoir 1 to 15μL.

(4) Set initial condition of wellplate reservoir 2 to 10μL.

(5) Set initial condition of wellplate reservoir 3 to 10μL.

(6) Add get tip operation.

(7) Add aspirate operation aspirating 3.24μL from wellplate reservoir 1.

(8) Add dispense operation dispensing 3.24μL into wellplate reservoir 3.

(9) Add remove tip operation.

(10) Add get tip operation.

(11) Add aspirate operation aspirating 1.15μL from wellplate reservoir 2.

(12) Add dispense operation dispensing 1.15μL into wellplate reservoir 3.

(13) Add mix operation mixing 10μL 5 times on wellplate reservoir 3.

(14) Add remove tip operation.

(15) BioCloneBot step is complete. Transfer contents of wellplate reservoir 3 into PCR tube and incubate at 50$\circ$C.

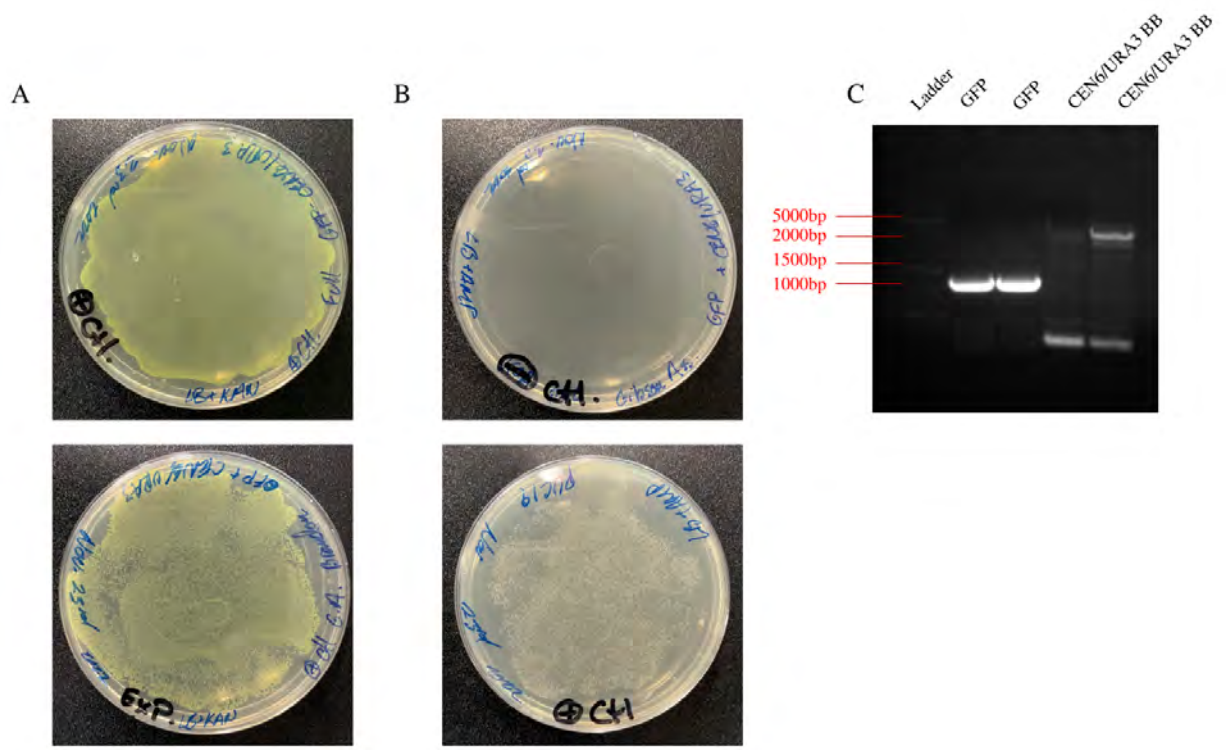


Figure 4.2: **Gibson Assembly of CEN6/URA3 GFP dropout plasmid using automated liquid handler. Reaction was done under the Hifi Assembly protocol where each fragment, GFP and CEN6/URA3 Backbone were PCR amplified with specific 20 nucleotide overhangs. (a) Positive control manually done for CEN6/URA3 GFP assembly and assembly using automated liquid handler. (b) Negative control where construct is plated on Amp+ instead of Kan+, and positive transformation control using pUC19 plasmid with a transformation efficiency of 10E7. (c) PCR Amplified GFP (1008bp) in duplicates, lane 2 & 3, as well as CEN6/URA3 backbone (3747bp) in duplicates, lane 4 & 5.**

4.2.3 Golden Gate Cloning

A Golden Gate Cloning was performed to insert a promotor, mRuby gene, and terminator into a Kan+ plasmid for E. coli. Using the tubestand labware, the assembly consisted of four DNA parts. The 250μL syringe was used inside of the syringe pump. The pump aspirated then dispensed volumes between 3μL and 4μL into a final collection tube resulting in a total of 19μL. The 19μL tube was run through a thermocycler. The resulting DNA plasmid was transformed into E. coli cells then plated. After plating, a mini-prep was run and the resulting DNA was run through a gel electrophoresis for an initial confirmation. The final DNA was sent for sequencing and returned with 100% sequence match. The process for automating the liquid handling portion of the experiment in the BioCloneBot software is outlined below:

(1) Add tubestand labware to platform.

(2) Add tipbox labware to platform.

(3) Set initial condition of tube 1 to 10μL.

(4) Set initial condition of tube 2 to 10μL.

(5) Set initial condition of tube 3 to 10μL.

(6) Set initial condition of tube 4 to 10μL.

(7) Set initial condition of tube 5 to 5μL.

(8) The initial condition of tube 6 is automatically 0 μL.

(9) Add get tip operation.

(10) Add aspirate operation aspirating 4μL from tube 1.

(11) Add dispense operation dispensing 4μL into tube 6.

(12) Add remove tip operation.

(13) Add get tip operation.

(14) Add aspirate operation aspirating 4μL from tube 2.

(15) Add dispense operation dispensing 4μL into tube 6.

(16) Add get tip operation.

(17) Add aspirate operation aspirating 4μL from tube 1.

(18) Add dispense operation dispensing 4μL into tube 6.

(19) Add remove tip operation.

(20) Add get tip operation.

(21) Add aspirate operation aspirating 3μL from tube 1.

(22) Add dispense operation dispensing 3μL into tube 6.

(23) Add remove tip operation.

(24) Add get tip operation.

(25) Add aspirate operation aspirating 4μL from tube 1.

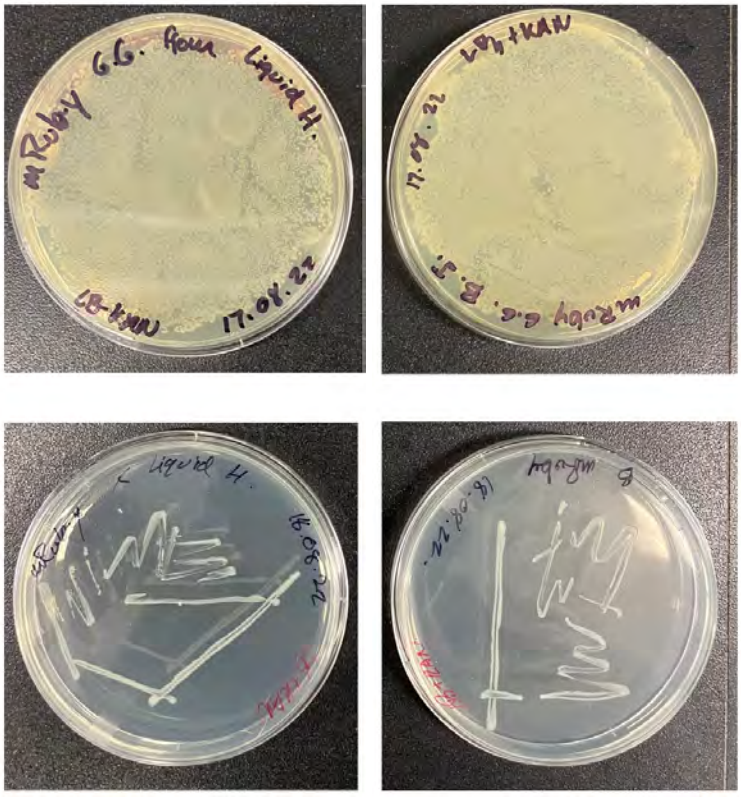
(26) Add dispense operation dispensing 4μL into tube 6.

(27) Add remove tip operation.

(28) Add mix operation mixing 10μL 5 times on tube 6.

(29) Add remove tip operation.

(30) BioCloneBot step is complete. Transfer contents of tube 6 into PCR tube and run amplification.



(a) (b)

Figure 4.3: **Golden Gate Cloning assembly of mRuby construct using the BioCloneBot. Digestion and ligation using the NEB Golden Gate assembly kit and plasmid: pYTK011 (pPGK1), pYTK034 (mRuby), pYTK056 (tTDH1) and CEN6/URA3-GFP dropout backbone. (a) Assembly of construct using liquid handler. Transformation of golden gate reaction with pPGK1-mRuby-tTDH1 contruct into E. coli was plated on LB+Kanamycin. Re-streaked to and isolate single colony. (b) Assembly of construct done manually as positive control.**

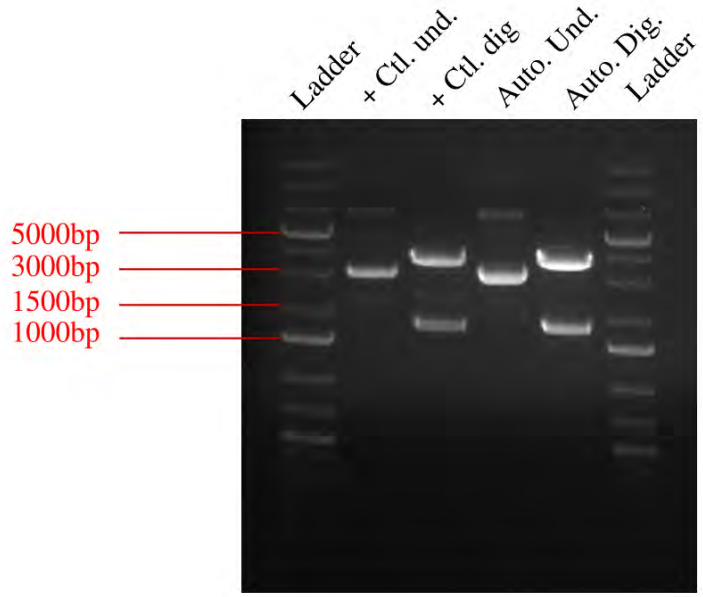


Figure 4.4: **Miniprep of isolated colonies from automated and manual assembly. The full length of assembly is 5733bp. Digestion with NotI results in bands at 3614bp and 1763bp.**

4.3 Biological Materials & Methods

4.3.1 Bacterial Strains, Plasmids, and Growth Conditions

Electrocompetent E. coli DH5-alpha cells were used in the transformation of all synthetically assembled DNA constructs through the liquid handler. Wildtype and mutant cells were all grown in Luria Bertani (LB) broth medium or on LB-agar plates at 37°C degrees celsius. The selection marker for the assembled plasmids was Kanamycin [38] and the medium was prepared with a working concentration of 50μg/mL. The media contained tryptone (10g/L), yeast extract (5g/L) and sodium chloride (10g/L), with the addition of 1.5% agar for plates.

Cells were made competent through the electrocompetent transformation protocol [39]. Cells were snap-frozen in liquid nitrogen and stored at -80°C until usage. Transformation of cells was done with the MicropulserTM [40] at 2.5kV for 4-6 milliseconds in 1cm electroporation cuvettes.

All plasmids were derived from the CEN6/URA3 Kan+ with a GFP dropout insert. The plasmid is a bacterial shuttle vector with a kanamycin-resistant marker, selectable in E. coli and a centromeric region for

replication in S. cerevisiae with a uracil auxotrophic selectable marker.

4.3.2 Cloning Automation Method I: Restriction Enzyme Digest and Ligation

The pCEN6/URA3 Kan+ GFP dropout plasmid was digested with BsaI (Eco31I) Type II restriction enzyme [41]. Liquid handler aspirated and dispensed volumes between 2-10μL for each segment: pCEN6/URA3 Kan+ GFP dropout (500ng), BsaI enzyme, 10X Buffer G and dH_2O. The final reaction tube was placed in a 37°C incubator for 2 hours to allow full digestion. After digestion, DNA was manually gel extracted with the GeneJet gel extraction kit [42] to separate pCEN6/URA3 backbone from GFP insert. The liquid handler aspirated and dispensed volumes between 2-10μL for each segment: pCEN6/URA3, GFP, T4 DNA ligase, 10X T4 DNA ligase Buffer [43]. The final reaction tube was placed at room temperature for 2 hours to allow full ligation. The ligated DNA fragments were manually transformed into electrocompetent E. coli DH5-alpha cells with the previously mentioned protocol. Cells were plated on Kanamycin-LB agar plates. Visualization of DNA was done on a 30mL 0.8% agarose gel with 2μL of 10mg/mL ethidium bromide.

4.3.3 Cloning Automation Method II: Gibson Assembly

Figure 4.5: **Theoretical and experimental design of each insert used in Gibson Assembly, where the top fragment illustrated the promoter region, GFP and terminator region and the bottom fragment represents the CEN6/URA3 backbone. Sequenced data demonstrated a 99.98% match between theoretical design and assembled construct. A deletion appeared in a non-coding region of the assembly plasmid.**

Two PCR products were amplified from the pCEN6/URA3 Kan+ GFP dropout backbone. The GFP fragment of 1030 base pairs and the pCEN6/URA3 backbone of 3530 base pairs were amplified with primers

designed to add a sequence that is 20 base pairs long and is identical in both fragments amplified, flanking the 5' and 3' sites. Primers were designed using the Benchling Gibson Assembly wizard software. Primers and DNA sequences can be found in the appendix D. The PCR products were amplified using the Phusion High Fidelity DNA polymerase [44]. The reaction mix contained 10uM dNTPs, 10X GC Buffer, forward and reverse primers at 1uM, 50ng of DNA template and water. The PCR reaction followed the protocol described by NEB for Phusion polymerase amplification with an annealing temperature of 54°C and an extension time of 30sec/kb. Two separate PCR reactions were performed to amplify the GFP segment and the pCEN6/URA3 backbone. Both fragments were amplified with an annealing temperature of 54°C and an extension time of 30 seconds per KB over 30 cycles. The PCR reactions were completed per segment to allow sufficient DNA to be collected following extraction with the GeneJET Gel Extraction Kit [42]. Visualization of DNA was done on a 30mL 0.8% agarose gel with 2μL of 10mg/mL ethidium bromide. The Hifi DNA Assembly Master Mix [45] was used to ligate the gel extracted GFP insert and pCEN6/URA3 backbone. Liquid handler aspirated and dispensed volumes between 2μL and 10μL for each segment and into a final reaction tube. The insert-to-vector ratio was 2:1, respectively, and the reaction mix was then placed in a thermocycler for 15 minutes at 50°C. Following the ligation, the assembled construct was transformed into electrocompetent E. coli DH5-alpha cells, as previously described, and plated for the selection of LB-agar Kan+ plates. Green fluorescence of the colonies confirmed successful assembly and GFP expression.

4.3.4 Cloning Automation Method III: Golden Gate Cloning and Moclo Toolkit

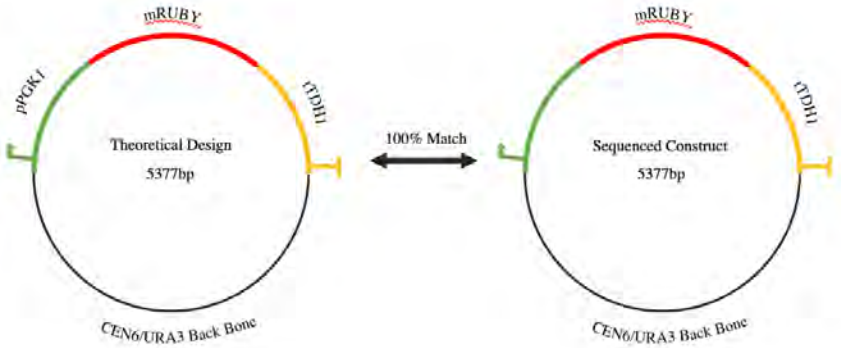


Figure 4.6: **Theoretical and experimental assembly of each insert for Golden Gate assembly, where forward arrow on pPGK1 fragment illustrates the promoter region, the middle mRuby insert illustrates gene of interest to express and the cross on the last tTDH1 fragment illustrates the terminator region. All inserts originated from the molecular cloning plasmids pYTK011 (pPGK1), pYTK034 (mRuby), pYTK056 (tTDH1) with the CEN6/URA3 backbone. Sequenced data demonstrated a 100% match between theoretical design and assembled construct. All sequences and primers can be found in the supplementary section of the thesis.**

Three inserts from the molecular Golden Gate toolkit [46] were used for assembly into the pCEN6/URA3 backbone. The final construct consisted of the promoter pPGK1 from the pYTK011 plasmid, the gene mRuby from the pYTK034 plasmid, the terminator tTDH1 from the pYTK056 plasmid and the pCEN6/URA3 backbone. The Golden Gate enzyme mastermix (NEB, E1601) was used in the positive control and experimental assembly of all 4 DNA segments. The positive control consisted of a manual assembly of the mRuby plasmid. The liquid handler aspirated and dispensed volumes between 2μL and 10μL for each segment and into a final reaction tube: pYTK011, pYTK034, pYTK056, NEB Golden Gate Mastermix. The final reaction tube was placed in a thermocycler with unpublished and proprietary conditions. Following golden gate assembly, half of the reaction (10μL) was manually transformed into electrocompetent E. coli DH5-alpha cells with the previously mentioned protocol. Cells were plated on kanamycin-LB agar plates. After 24 hours of incubation at 37°C, 1 colony from the positive control plate and 1 colony from the automated experimental plate were isolated and restreaked onto kanamycin-LB agar plates. Half of each colony was inoculated in 10mL LB media with 10μL of 50mg/mL kanamycin, shaking overnight at 200rpm at 37°C. Following 24 hours incubation, the liquid cultures of both underwent a miniprep with the GeneJet Plasmid

Miniprep kit [47]. The DNA extracted was digested with NotI 10U/μL [48]: 500ng of each combined with 0.5μL enzyme, 10X Buffer and incubated at 37°C degrees for 2 hours. Visualization of DNA was done on a 30mL 0.8% agarose gel with 2μL of 10mg/mL ethidium bromide.

Chapter 5

Conclusion & Future Work

Automation is increasingly used in the field of synthetic biology for automating commonly performed experiments such as PCR and DNA assembly. To further increase accessibility to automation equipment, cheap alternatives are being developed.

In this thesis, a low-cost open-source automation device has been developed, characterized, and tested. The total cost of the BioCloneBot falls below $\$1,500$ CAD including all required parts. A detailed bill of materials can be found in C. To accomplish partial automation of DNA assembly experiments, a custom re-usable microliter syringe pump was developed. The results show that the syringe pump is capable of accurately and precisely moving volumes between 1μL and 200μL. As lab automation becomes more affordable and available, more scientists will be able to reduce the amount of time spent performing repetitive liquid handling operations. Devices such as the BioCloneBot make of lab automation more available to researchers with small budgets. The following tables compare the BioCloneBot to the previously reviewed automated liquid handlers. Since each reviewed evice was not tested using the same volumes, the closest related volumes were chosen.

Device	Creator	Supported Volume Range	Minimum Cost (CAD)	Open-Source Software	Open-Source Hardware
epMotion 5075t	Eppendorf	1μL-1,000μL	$\$110,000$	No	No
Microlab NIMBUS4	Hamilton	1μL-1,000μL	$\$35,000$	No	No
Biomek 4000	Beckman Coulter	1μL-1,000μL	$\$20,000$ used	No	No
OpenTrons-OT2	OpenTrons	1μL-1,000μL	$\$8,736$	Apps and protocols only	Designs only
EvoBot	Faiña et al. [26]	10μL-10,000μL	$\$806$	Yes	Yes
OTTO	Florian et al. [32]	NA	$\$2,015$	Yes	Yes
BioCloneBot	Wells, Ke'Koa	1μL-200μL	$\$2,015$ \$	Yes	Yes

Table 5.1: **Comparison of BioCloneBot against reviewed liquid handler devices.**

Device	Add-Ons
epMotion 5075	Multiple pipettors, ThermoMixer (included), gripper (included)
Microlab NIMBUS4	Multiple Pipettors, labware gripper, barcode scanner, shft-n-scan tube barcode scanner, CO-RE paddles, $[MPE]^2$ positive pressure solid phase extraction and evaporation module, CO-RE lid tool, vacuum station, clean air system, heater shaker, plate tilt module, fill module
Biomek 4000	Multiple pipettors, ALP labware stand, tube rack ALP, auto-latch tip rack holders, orbital shaker, disposal accessory, liquid waste station, bar code reader, circulating reservoir, filtration system, off-deck platform, thermal exchange unit, wash system
OpenTrons-OT2	Multiple pipettors, thermocycler, temperature module, magnetic module, HEPA module, heater-shaker module
EvoBot	Multiple pipettors, dispensing module, heavy payload module
OTTO	None, but could accommodate
BioCloneBot	Supports the future inclusion of a thermocycler

Table 5.2: **Table of automated liquid handlers' vs BioCloneBot add-ons.**

Device	Volume	Accuracy	Precision
epMotion 5075	1μL	$\pm$20.0%	$\pm$5.0%
epMotion 5075	5μL	$\pm$5.0%	$\pm$3.0%
epMotion 5075	25μL	$\pm$1.5%	$\pm$0.6%
epMotion 5075	50μL	$\pm$1.0%	$\pm$0.4%
epMotion 5075	100μL	$\pm$2.0%	$\pm$1.0%
epMotion 5075	200μL	$\pm$0.6%	$\pm$0.3%
Microlab NIMBUS4	1μL	$\pm$5.0%	$\pm$5.0%
Microlab NIMBUS4	5μL	$\pm$2.5%	$\pm$2.0%
Microlab NIMBUS4	25μL	NA	NA
Microlab NIMBUS4	50μL	$\pm$2.0%	$\pm$1.0%
Microlab NIMBUS4	100μL	$\pm$2.0%	$\pm$1.0%
Microlab NIMBUS4	200μL	$\pm$1.0%	$\pm$1.0%
Biomek 4000	NA	NA	NA
OpenTrons-OT2	1μL	$\pm$20.0%	$\pm$5.0%
OpenTrons-OT2	5μL	NA	NA
OpenTrons-OT2	20μL	$\pm$1.5%	$\pm$0.8%
OpenTrons-OT2	50μL	NA	NA
OpenTrons-OT2	100μL	$\pm$2.0%	$\pm$1.0%
OpenTrons-OT2	300μL	$\pm$0.6%	$\pm$0.3%
EvoBot	NA	NA	NA
OTTO	NA	NA	NA
BioCloneBot	1μL	$\pm$5.0%	$\pm$11.22%
BioCloneBot	5μL	$\pm$2.25%	$\pm$12.50%
BioCloneBot	20μL	$\pm$1.05%	$\pm$0.97%
BioCloneBot	50μL	$\pm$0.74%	$\pm$0.39%
BioCloneBot	100μL	$\pm$0.93%	$\pm$0.36%
BioCloneBot	200μL	$\pm$0.95%	$\pm$0.32%

Table 5.3: **Comparison BioCloneBot against automated liquid handlers' pipettor accuracy and precision.**

5.1 Future Work

Given the vast scope and the DIY nature of the BioCloneBot, there are several improvements that can be made. Since the BioCloneBot covers aspects of mechanical, electrical, and software engineering, the future

work is broken down into these major sections. While this list is extensive, there are many improvements that are possible. This is the beauty of open-source projects such as the BioCloneBot.

5.1.1 Mechanical

Hamilton offers a range of microliter syringes from 0.5μL all the way to 500μL. Since the syringes have similar dimensions and use the same removable nut, more syringes could be tested to increase the range of volumes the system can handle.

Due to error introduced by 3D printed parts, missed targets are sometimes observed. A small dimensional error of 0.1mm or 0.2mm can be enough for the syringe pump to miss the liquid inside of a target labware. As an example, if the target is a 5mL Eppendorf tube or 96-wellplate containing a volume of 10μL or less, a small dimensions variation of 0.1 or 0.2mm can cause the pump to miss resulting in a failed aspiration.

The standard microliter Hamilton syringes were not designed to be run dry. As such, the plunger needs to be lubricated using water before every experiment otherwise the syringe does not have a proper seal. This can result in a significant amount of leakage from a well-attached pipette tip.

With the current design, the syringe pump greatly struggles with volumes under 1.0μL. It is most likely caused by error introduced by the mechanical design. Error introduced by backlash from the lead screw and lead screw nut may be causing this issue. Backlash occurs due to imperfect manufacturing. A lead screw advertised with a 2mm pitch, may actually have a pitch of 2.05mm. When the syringe pump changes directions, the lead screw nut has to travel an extra 0.05mm before the syringe plunger moves. Several syringes were tested, but the problem was consistent across each one meaning it is unlikely a problem related to the pressure or travel distance required by the syringe. In addition, over aspiration on the lower volumes could be a result of the capillary effect. As the diameter of a pipette tip gets smaller, the likelihood of observing the capillary effect increases. It occurs when the adhesion and cohesion forces between a liquid and a surface cause the liquid to move into a tube, cylinder, etc. without the introduction of an external force.

5.1.2 Electrical

Instead of using a breadboard, a custom PCB could be designed. Using custom PCBs drastically reduces the amount of wiring. The benefits of a PCB greatly outweigh the extra cost of PCB production. With less

wiring required by the user, there is a significantly reduced chance of injury due to electrical shock and a reduced chance of damaging the electronics due to miswiring. It also allows for the creation of an electrical housing. This allows for the implementation of physical controls such as an emergency stop button as well as electronics for interfacing with the microcontroller without modifying the firmware or using the front-end software. The circuit would also benefit from protection from voltage spikes or over-drawn current.

The speed of the 3-axis system is not on par with the commercial equivalents. To resolve this, a faster microcontroller and different motor drivers could be used.

5.1.3 Software

The following software improvements include both the firmware and the front-end code.

- Implementation of acceleration/deceleration for each stepper motor to increase speed while maintaining accuracy
- Ability to delete specific operations in a user-created protocol
- Add time estimation of a liquid handling protocol
- Include the ability to cancel liquid handling protocols without using the power supply switch
- Add the ability to select which syringe is used in the front-end which can update the values on the microcontroller
- Implementation of the Ender 3 Pro LCD to the microcontroller firmware
- Overall improvement the labware/operation object-oriented implementation for easier user
- Functionality to add new types of labware into the software directly without modifying the code

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[48] "NotI (10 u/µl)." (), [Online]. Available: https://www.thermofisher.com/order/catalog/product/ER0592 (visited on 12/10/2022).

[49] "Pinout for the standard ATX 24-pin 12v motherboard power connector," Lifewire. Section: Lifewire. (), [Online]. Available: https://www.lifewire.com/atx-24-pin-12v-power-supply-pinout-2624578 (visited on 12/11/2022).

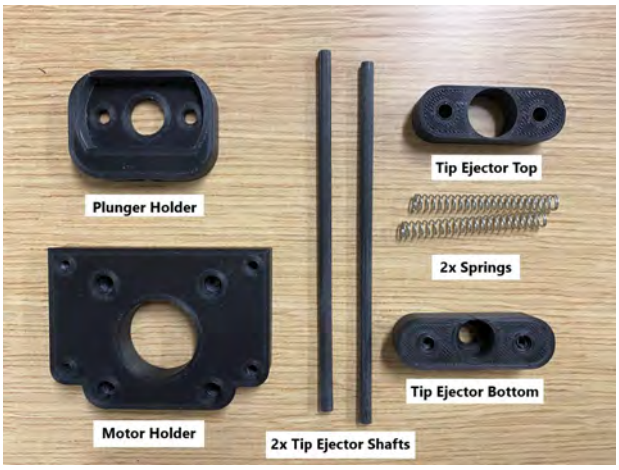
Appendix A

Assembly Guide

The assembly guide, software, guide, BOM, and code base can be accessible through the following GitHub repo: BioCloneBot Github

A.1 Syringe Pump Assembly

(a)



(b)

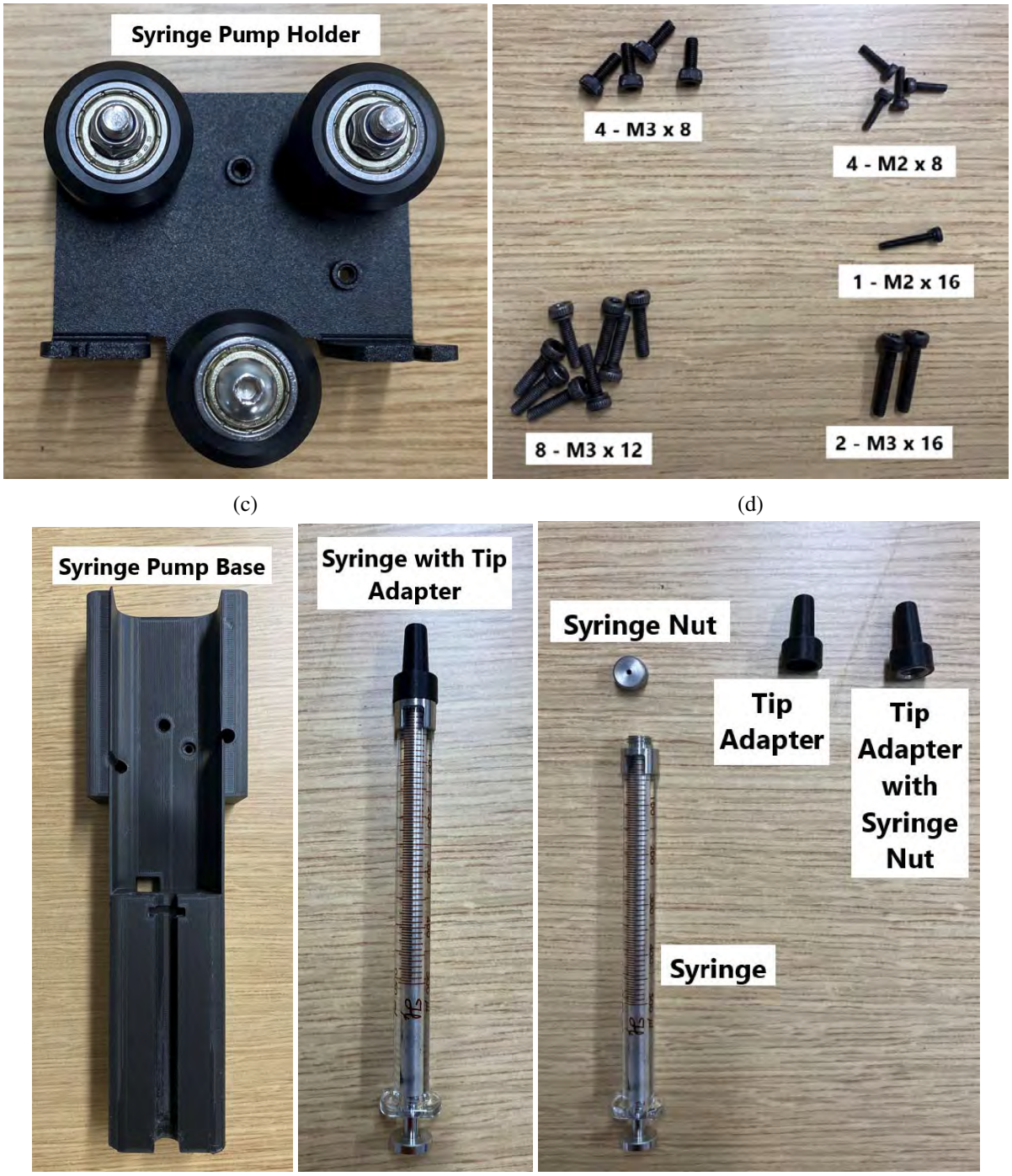


Figure A.1: **Syringe Pump Components**

The Syringe Pump Holder comes from disassembling the Ender 3 Pro in the later steps. Attach the Syringe Pump Holder to the Syringe Pump Base using four M3x12 screws. The print in the picture is

missing the 4th screw hole due to a 3D printing error. Your pump base should have four holes.

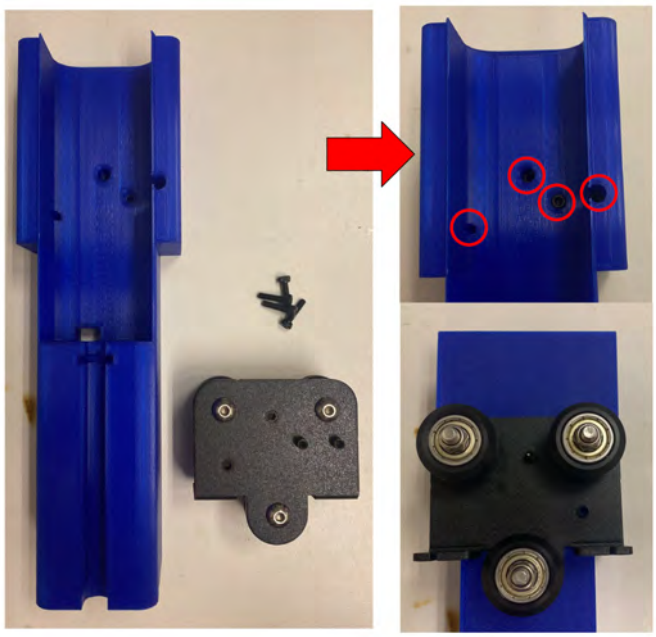


Figure A.2: **Syringe Pump Base attachment to Syringe Pump Holder.**

Attach the Syringe Pump Stepper Motor to the Motor Holder using four M3x8 screws.

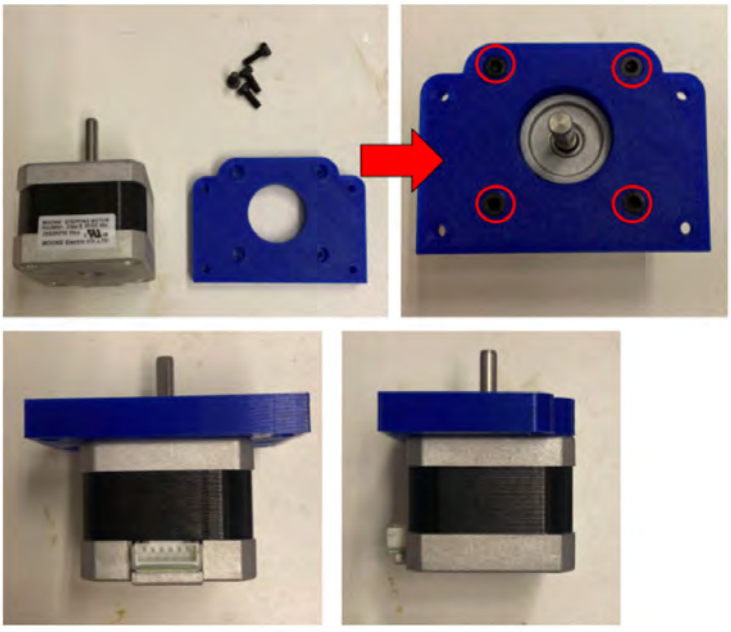


Figure A.3: **Pump Stepper Motor attachment to Motor Holder.**

Attach the Shaft Coupler to the Lead Screw using the two set screws. Attach the Shaft Coupler to the stepper motor shaft using the last two set screws. It's important to attach the Lead Screw first so the motor shaft fits the Shaft Coupler properly.

Figure A.4: **Lead Screw attachment to Pump Stepper Motor.**

Attach the Lead Screw Nut to the Plunger Holder using two M2x12 screws, two M2 nuts, and two M2 washers. Note that the Lead Screw Nut should fit very tightly - it will require some force to push it all the way in.

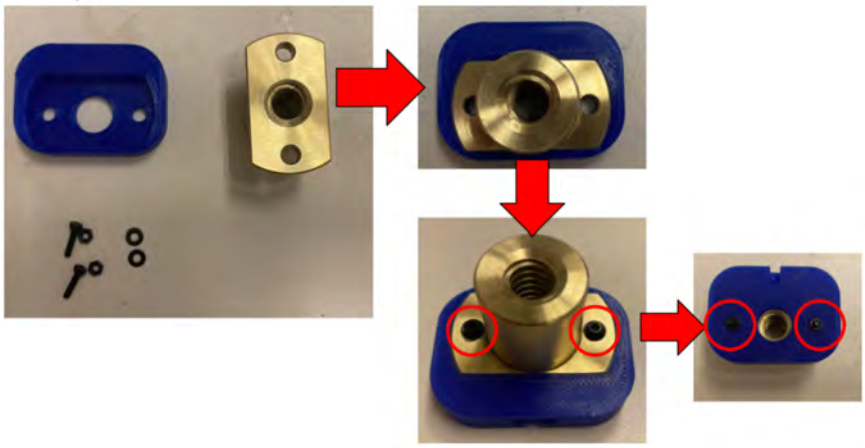


Figure A.5: **Lead Screw Nut attachment to Plunger Holder.**

Now we will assemble the Tip Ejector. Screw on two of the Tip Ejector Shafts to the Tip Ejector Top using

two M2x8 screws. Then slide the two springs around the shafts. If the springs do not fit smoothly, sand down the shafts until the spring moves easily with little to no resistance.

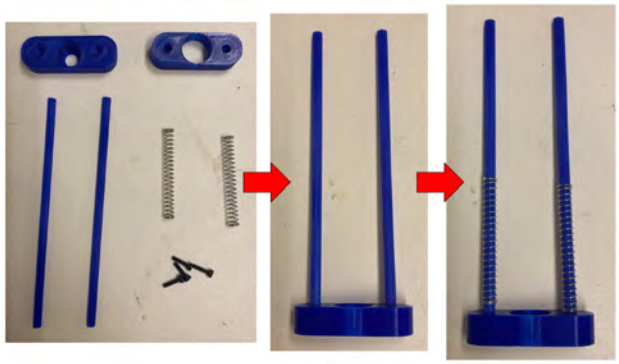


Figure A.6: **Tip Ejector preparation.**

Next, slot the syringe into the Syringe Pump Base. Insert the shafts into the Syringe Pump Base, then compress the springs with your hand. With the springs compressed, screw the Tip Ejector Bottom to the Tip Ejector Shafts using two M2x8 screws. NOTE: The Tip Ejector Bottom is not symmetrical - if it does not fit flush against the Syringe Pump Base, try rotating it 180 degrees.

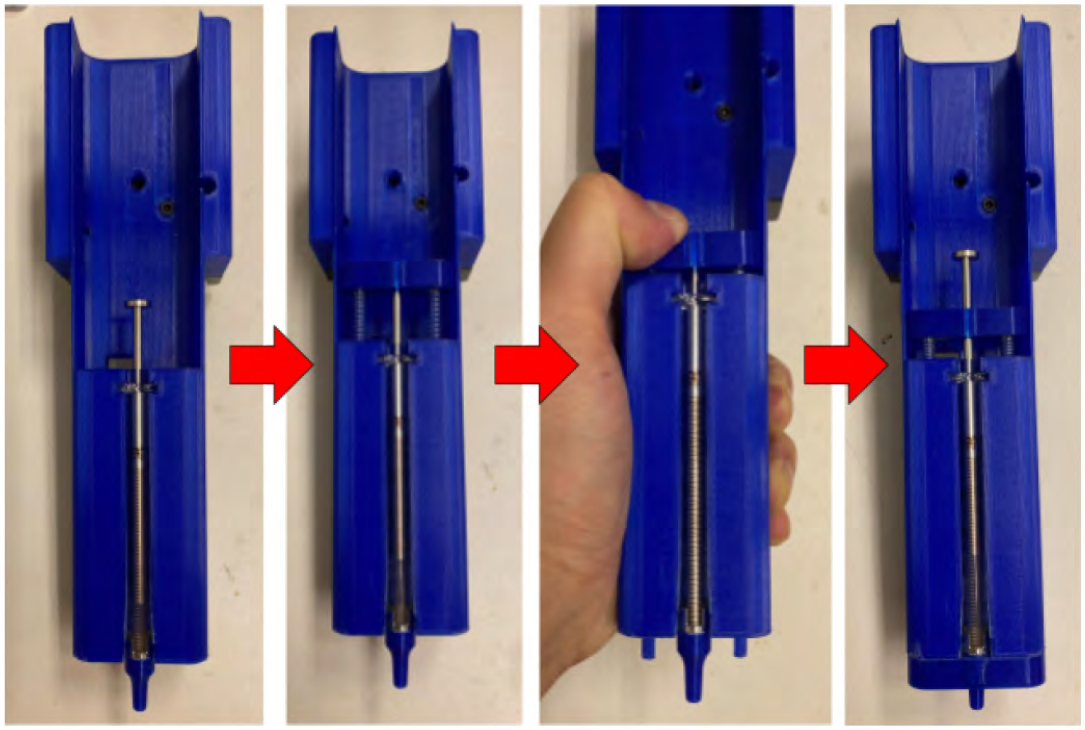


Figure A.7: **Syringe and Tip Ejector attachment.**

Remove the Syringe Plunger and slot it into the Plunger Holder. Next, slide the Syringe Plunger back into the Syringe. Line up the Lead Screw with the Lead Screw Nut. You can rotate the Shaft Coupler to thread the Lead Screw into the Lead Screw Nut.

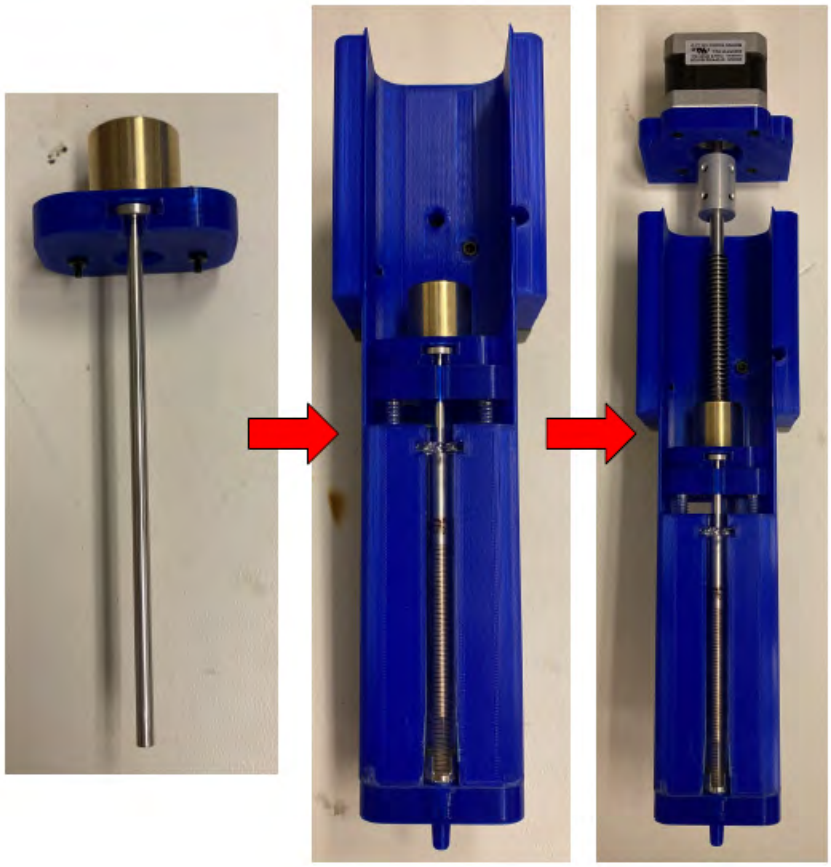


Figure A.8: **Syringe Plunger and Lead Screw attachment.**

Continue screwing in the Lead Screw until the Motor Holder is flush with the Syringe Pump Base. Screw the Motor Holder to the Syringe Pump base using four M3x8 screws.

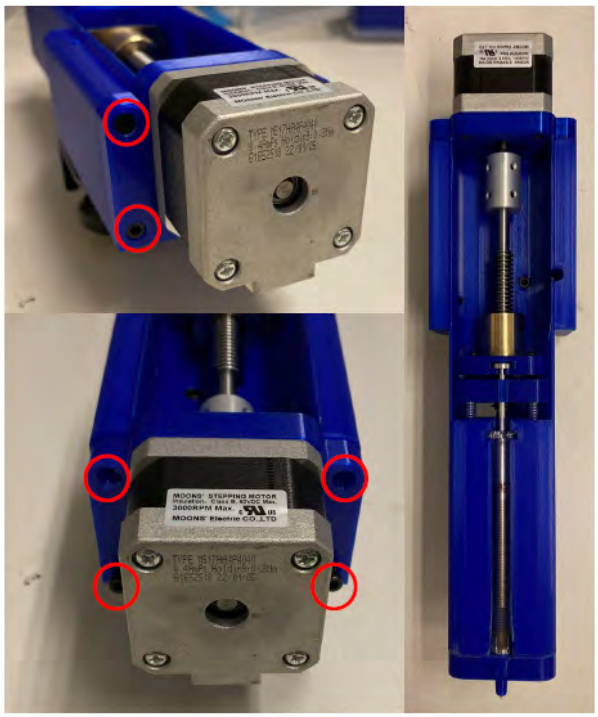


Figure A.9: **Pump Motor attachment.**

Next solder two wires to the Pump Limit Switch. Solder one wire to the NC terminal and one wire to the COM terminal on the limit switch. The limit switch may be different than the original picture, but that is OK. Both are identical in size and the mounting holes are the same. They both also include the same three terminals.

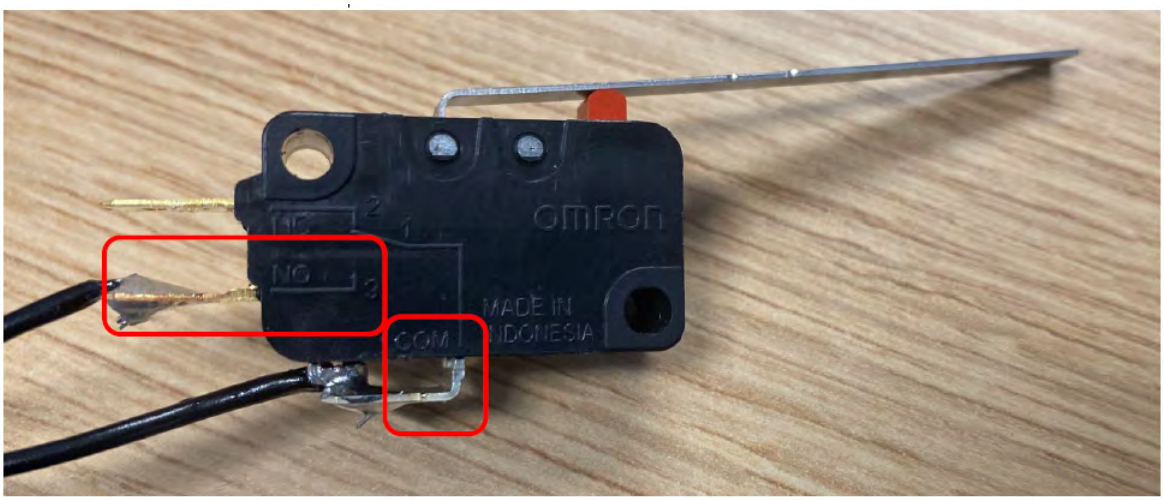


Figure A.10: **Pump Limit Switch soldering.**

The last step is to insert the Pump Limit Switch into the Syringe Pump Base and fasten it using two M3x16 screws.

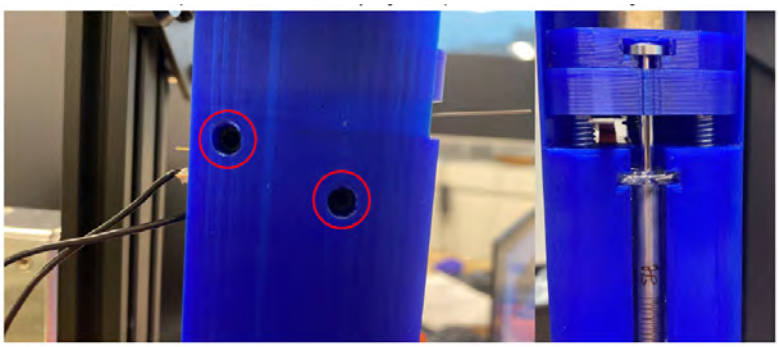


Figure A.11: **Pump Limit Switch attachment.**

A.2 Frame Assembly

To begin, we will assemble the frame of the BioCloneBot. Start by opening the Ender 3 Pro box and removing all of the parts.

Figure A.12: **Ender 3 Pro Base**

Remove the four knobs from the bottom of the Ender 3 Pro Base A.13. Make sure not to lose the springs. Put the knobs and springs aside for later.

(a) (b)

Figure A.13: **Ender 3 Pro base knobs.**

Carefully remove the Ender 3 Pro bed and place it to the side.

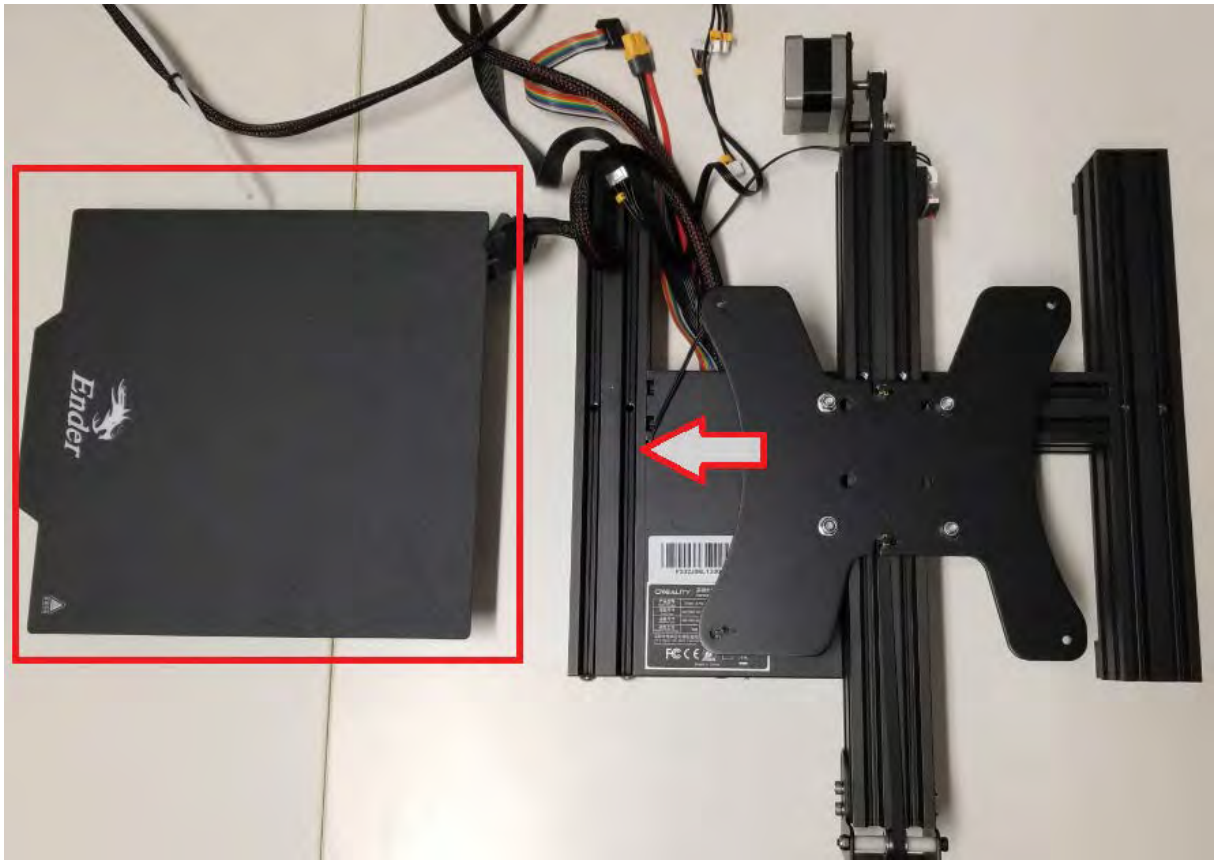


Figure A.14: **Ender 3 Pro Bed removal.**

Before removing the Y-Axis belt tensioner, feel how tight belt is. The belt needs to be approximately this tight when attaching the new larger Y-Axis belt from the Ender Xtender kit. Remove the four screws from the Y-Axis Belt Tensioner and place them to the side for later use.

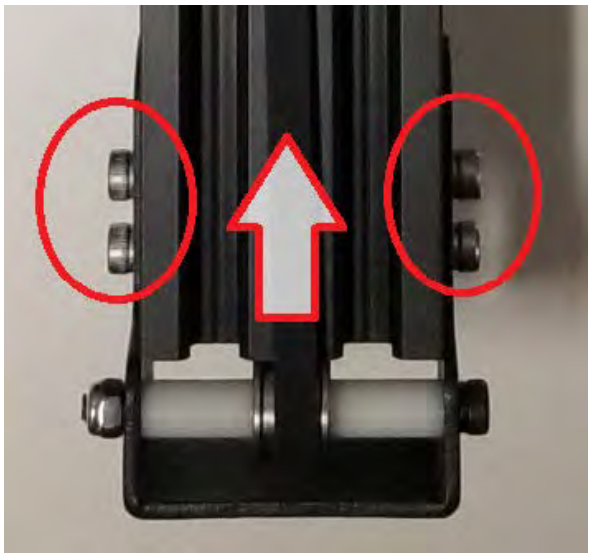
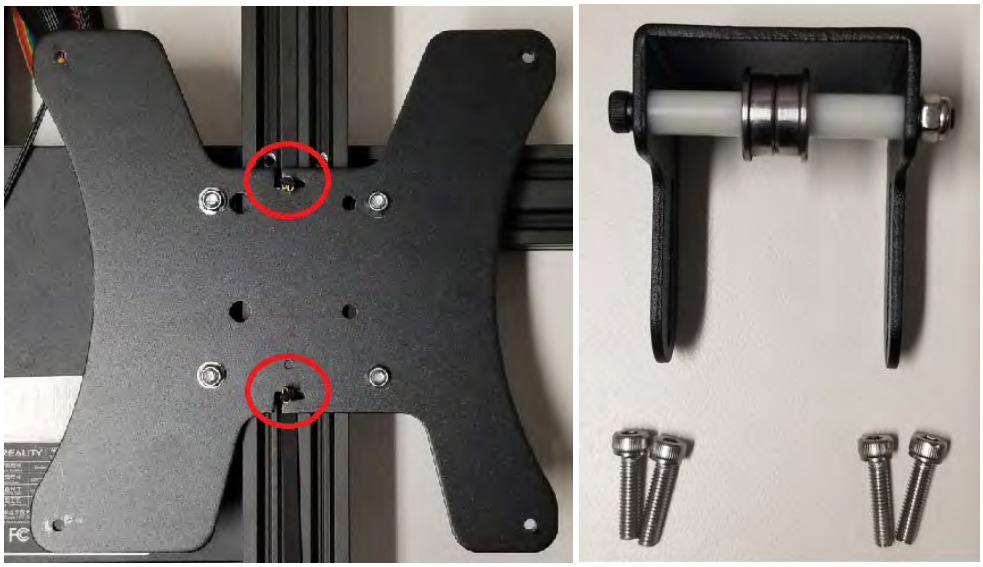


Figure A.15: **Y-Axis Belt Tensioner removal.**

Unhook both ends of the Y-Axis belt from the Y-Axis Carriage. Carefully remove the Y-Axis Belt Tensioner from A.16b. Place it with the four screws removed from earlier.



(a) (b)

Figure A.16: **Y-Axis Belt Tensioner removal.**

Remove the Y-Axis Carriage from the frame and place it aside for later.

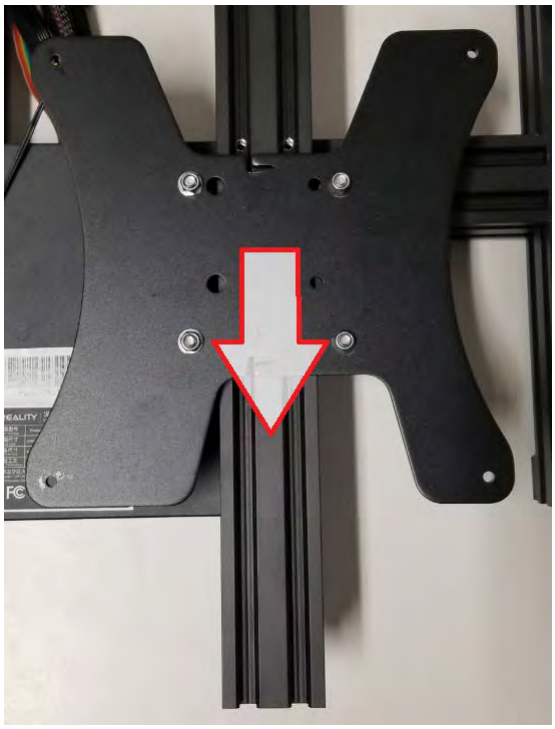
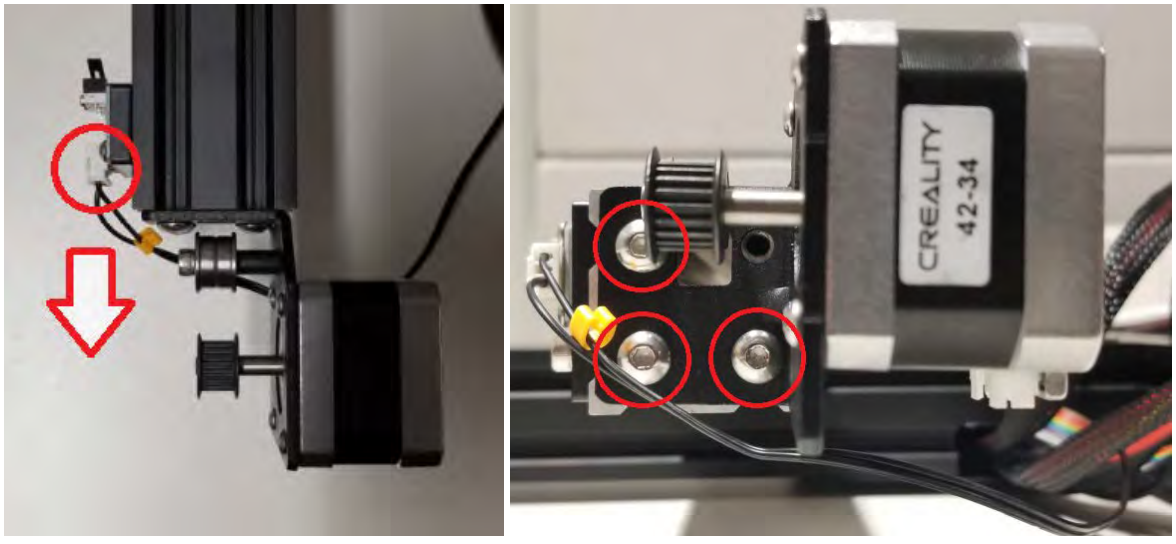


Figure A.17: **Y-Axis Carriage removal.**

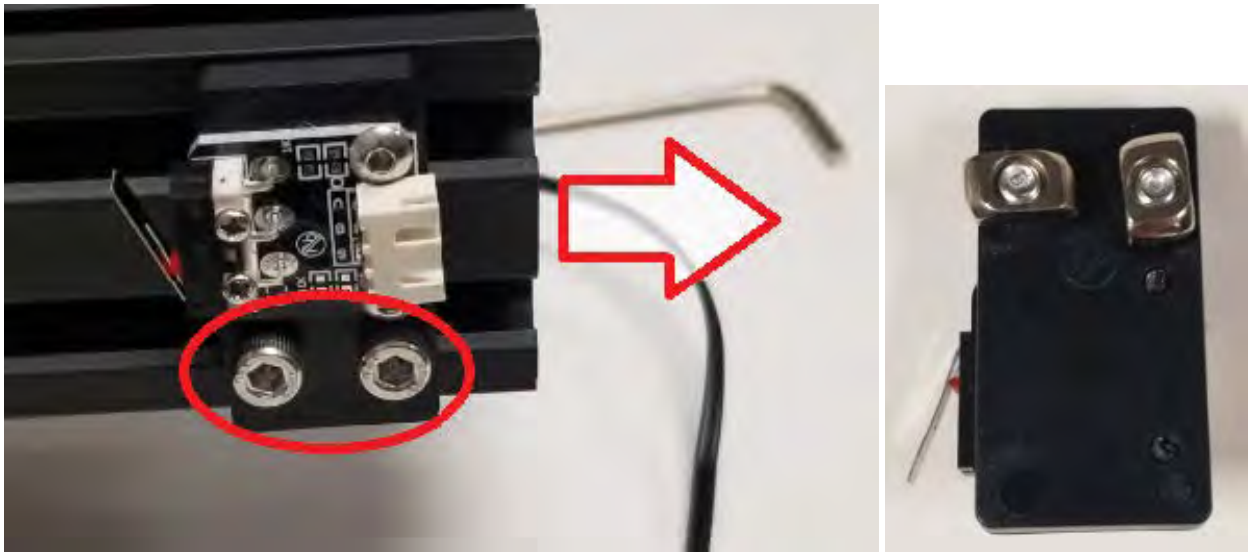
On the back of the base, unplug the Y-Axis Limit Switch Connector then unscrew the three screws holding the Y-Axis Stepper Motor. Put the Y-Axis Stepper Motor and the three screws aside for later.



(a) (b)

Figure A.18: **Y-Axis Stepper Motor removal.**

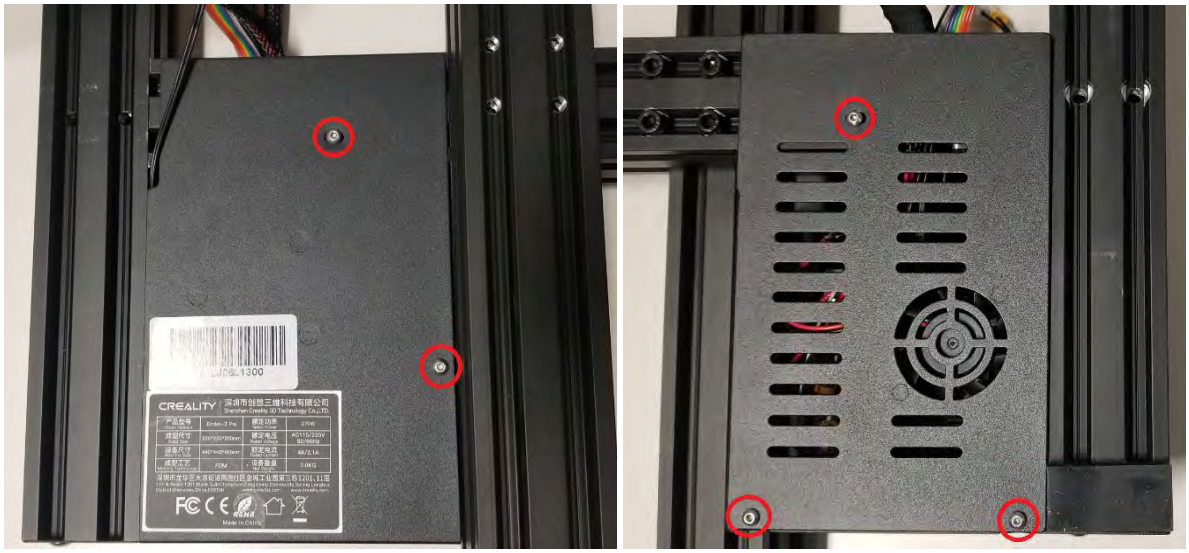
Remove Y-Axis Limit switch by unscrewing the two screws and sliding the switch off the extrusion. Place the limit switch, screws, and t-nuts aside for later.



(a) (b)

Figure A.19: **Y-Axis Limit Switch removal.**

Next we will remove the electronics housing. Remove the two screws from the bottom and the three screws from the top of the electronics housing.



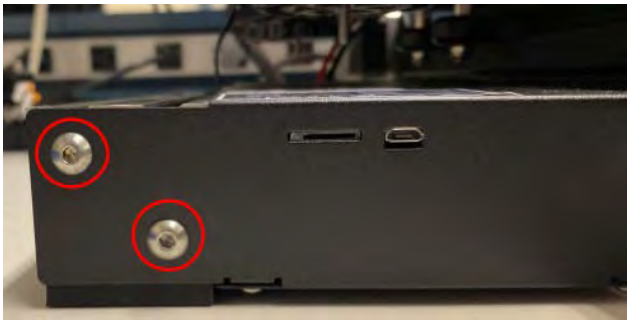
(a) (b)

Figure A.20: **Electronics Housing removal.**

Carefully remove the bottom cover of the housing. Unplug the fan before completely removing the cover.

Figure A.21: **Housing Fan removal.**

Remove the two screws from the front of the Electronics Housing. The housing will no longer be attached to the frame. Remove the wiring bundle from the notch in the bottom of the frame.



(a)

(b)

Figure A.22: **Electronics Housing removal.**

The remaining frame should look like this.

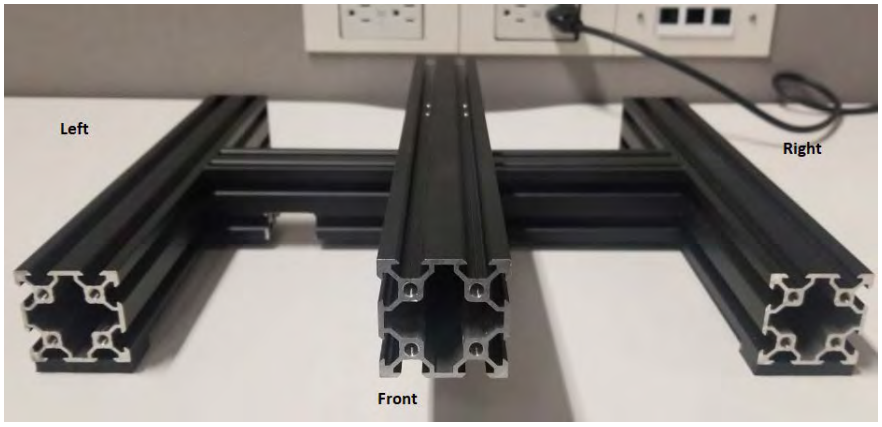
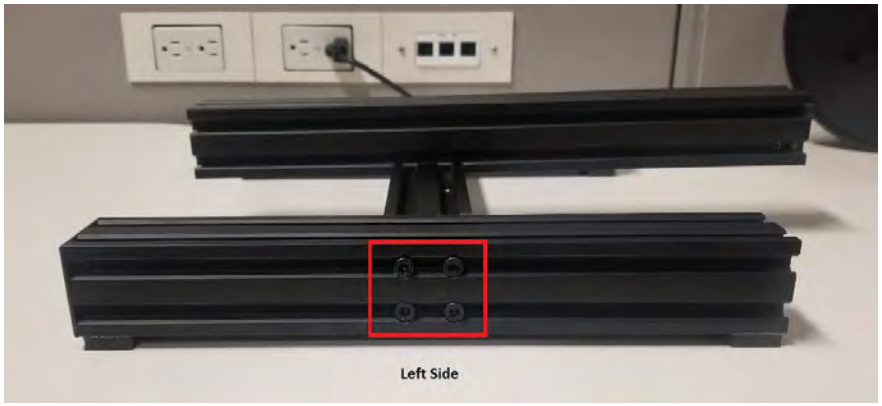


Figure A.23: **Ender 3 Partial Frame.**

Remove the eight screws from the left and right extrusions. There are four screws on each side.

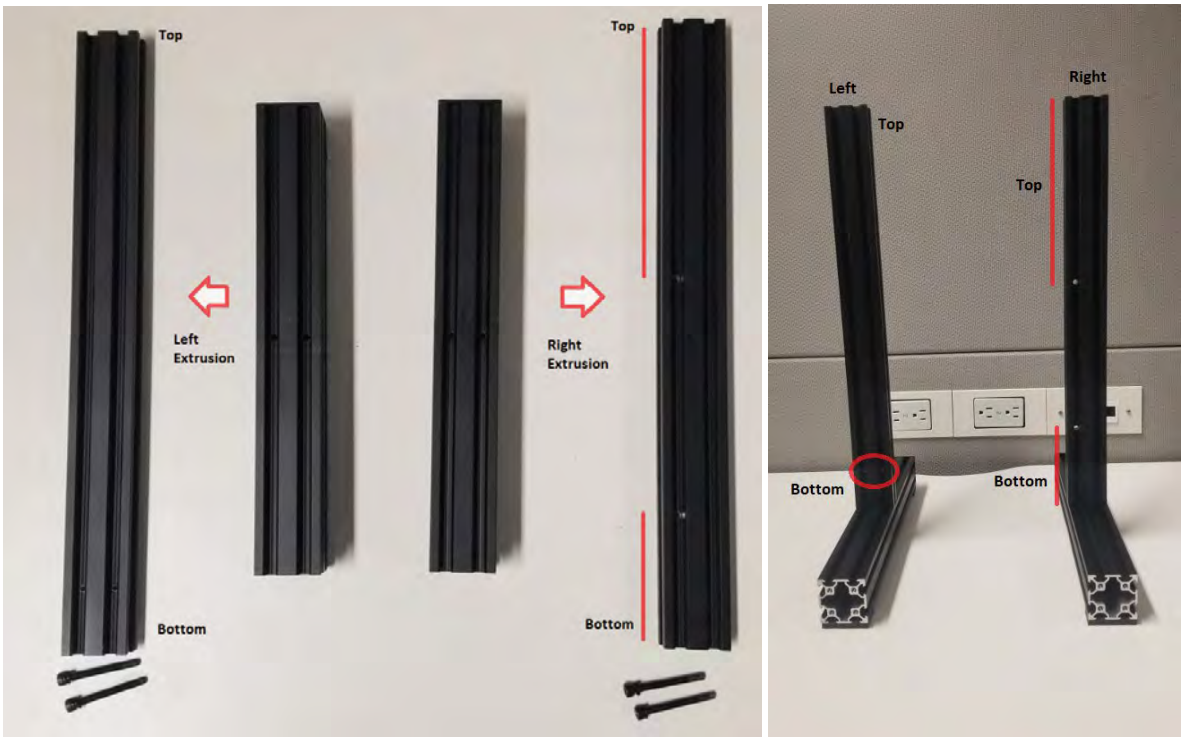
(a)



(b)

Figure A.24: **Partial frame side removal.**

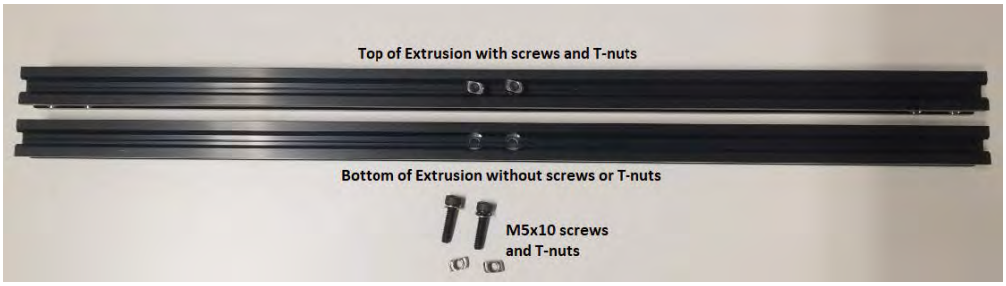
Next, install the Vertical Extrusions to the two side extrusions from the previous step using the the parts provided with the Ender 3 Pro. Each side requires two M4x16 screws.



(a) (b)

Figure A.25: **Ender 3 Pro Vertical Extrusion install.**

Grab the two Horizontal Extrusions, eight M5x25 screws and M5 star lock washers, two M5x10 screws, and two T-nuts from the Ender Xtender kit. Both extrusions are identical. Add two M5x15 screws to both of the Horizontal Extrusions as seen in the figure below. Using four of the M5x25 screws and M5 star washers, screw one extrusion to the front of the frame using the bottom sets of holes.



(a)

(b)

Figure A.26: **Ender 3 Pro Horizontal Front Extrusion install.**

Turn around the frame and remove the two end covers from the end of the extrusions.

Figure A.27: **Rear frame extrusion cover removal.**

Install the remaining extrusion to the bottom sets of holes on the rear of the frame using the remaining four M5x25 screws and M5 star lock washers.

Figure A.28: **Rear Horizontal Extrusion install.**

Grab the large Y-Axis Extrusion from the Ender Xtender kit. Using the T-nuts you previously installed to the Horizontal Extrusions, attach the Y-Axis Extrusion to the frame. Note that this part can be a little bit tricky. I like to use the small allen key included with the Ender 3 Pro to help line up the T-nuts properly.

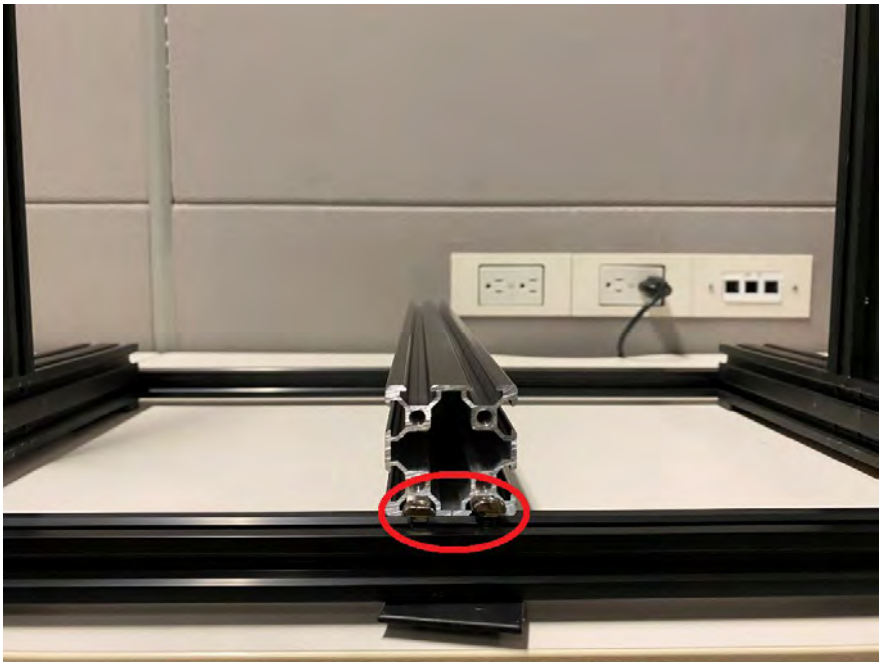


Figure A.29: **Y-Axis Extrusion install.**

Once the T-nuts are properly attached, use a ruler and offset the Y-Axis Extrusion by 170mm. Once aligned properly, tighten the T-nuts using the appropriate allen key from the Ender 3 Pro kit.

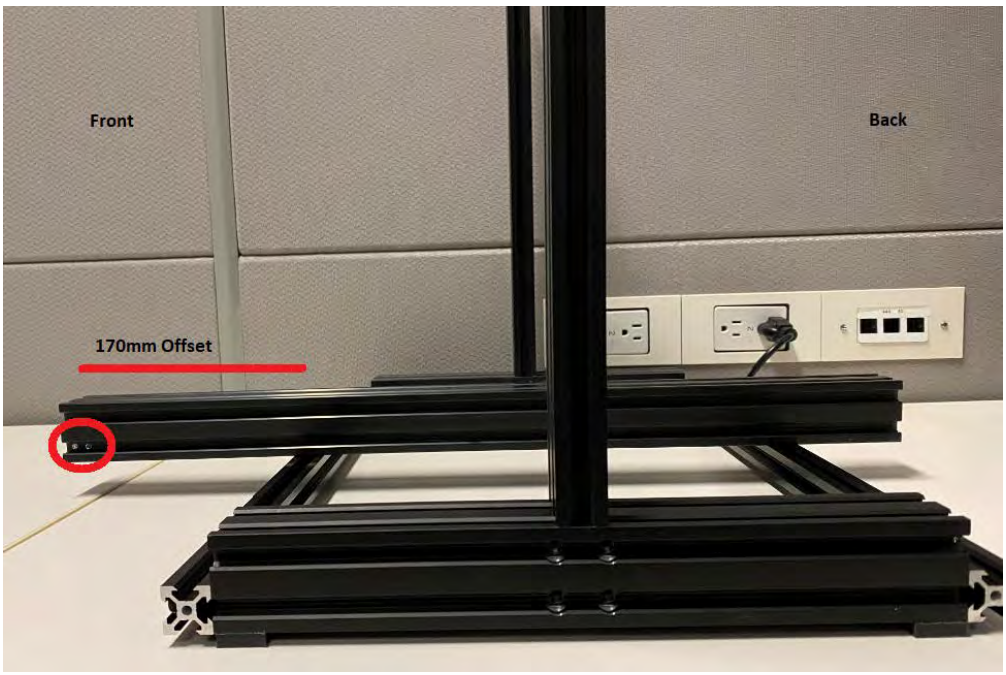


Figure A.30: **Y-Axis Extrusion alignment.**

Re-attach the Y-Axis Carriage ensuring that the thin corner is in the back left.

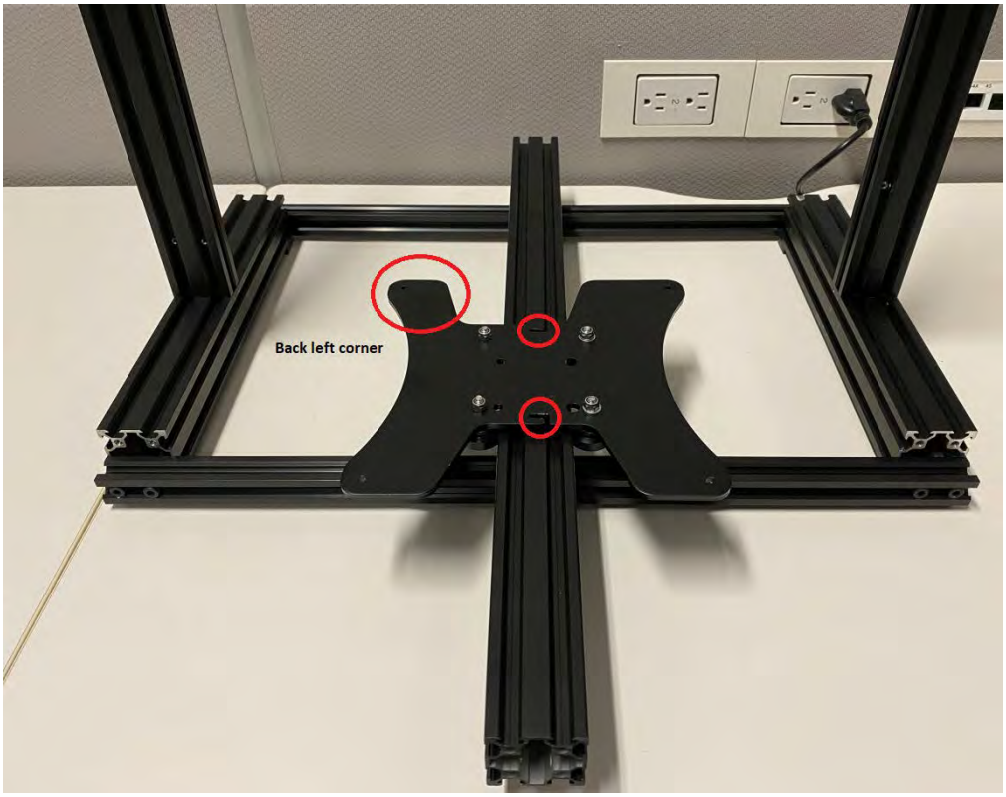


Figure A.31: **Y-Axis Carriage reattachment.**

Next, feed the Y-Axis Belt that comes with the Ender Xtender kit through the Y-Axis Extrusion.

(a) (b)

Figure A.32: **Ender Xtender Y-Axis Belt replacement.**

On the back left of the Y-Axis Extrusion, re-attach the Y-Axis Limit Switch using the two screws from A.19. Your screws should be silver (I lost the originals and had to use two different ones). Align the switch so it is flush with the end of the Y-Axis Extrusion.

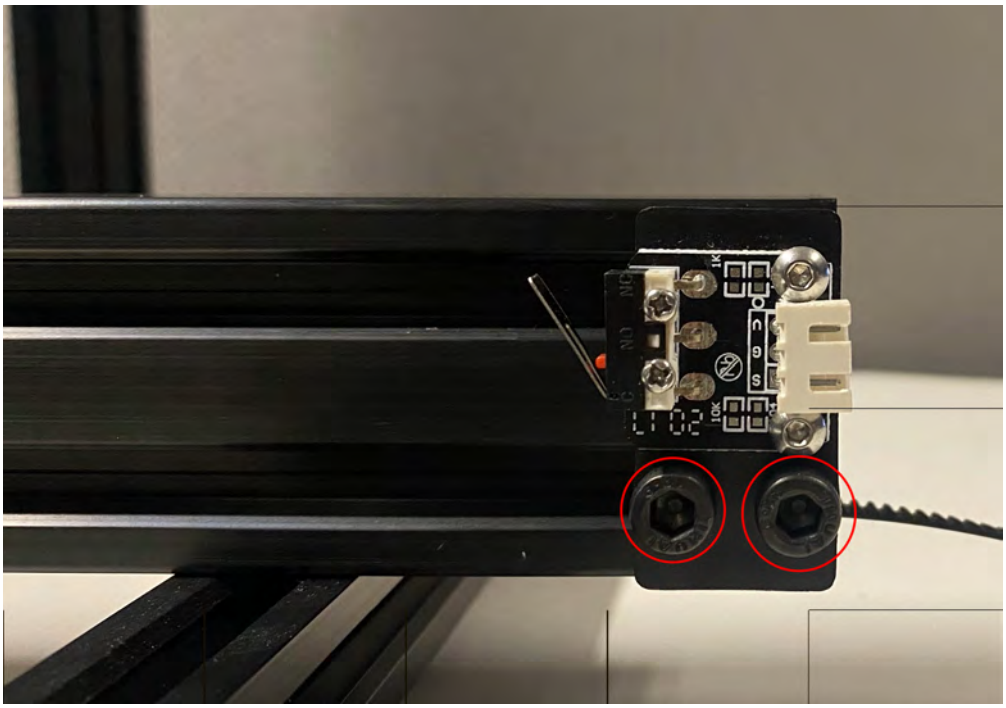


Figure A.33: **Y-Axis Limit Switch re-attachment.**

Feed the Y-Axis Belt through the hole Y-Axis Stepper Motor Assembly then re-attach the Y-Axis Stepper Motor Assembly with the three screws from step A.18.

(a) (b)

Figure A.34: **Y-Axis Stepper Motor Assembly re-attachment.**

Attach the Y-Axis Belt to the Y-Axis Carriage.

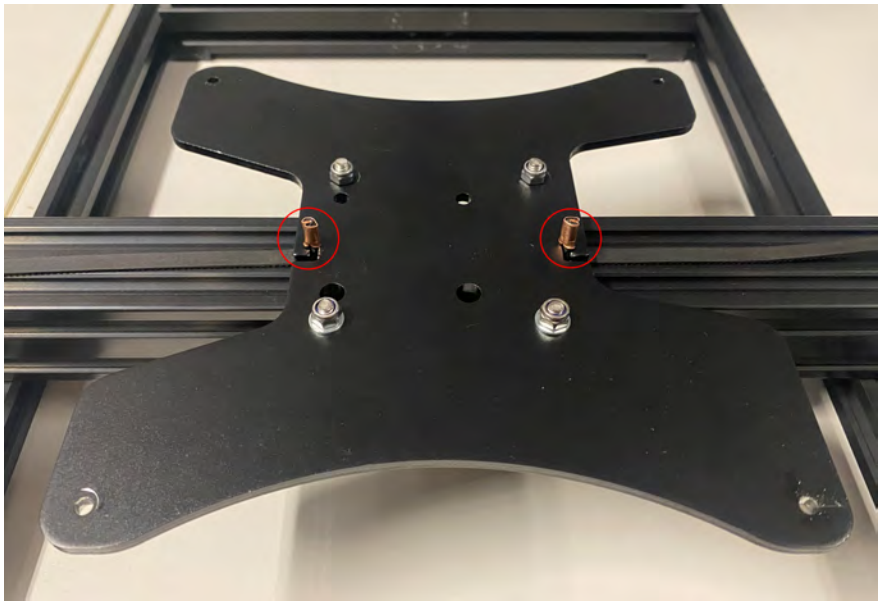


Figure A.35: **Y-Axis Belt re-attachment.**

Feed the Y-Axis Belt through the hole in the Y-Axis Tensionser, then re-attach the Y-Axis Tensioner using the by partially tightening two screws on each side from A.15. Once the all four screws are in, pull on the Y-Axis Belt Tensioner to tighten the belt and firmly tighten the screws.

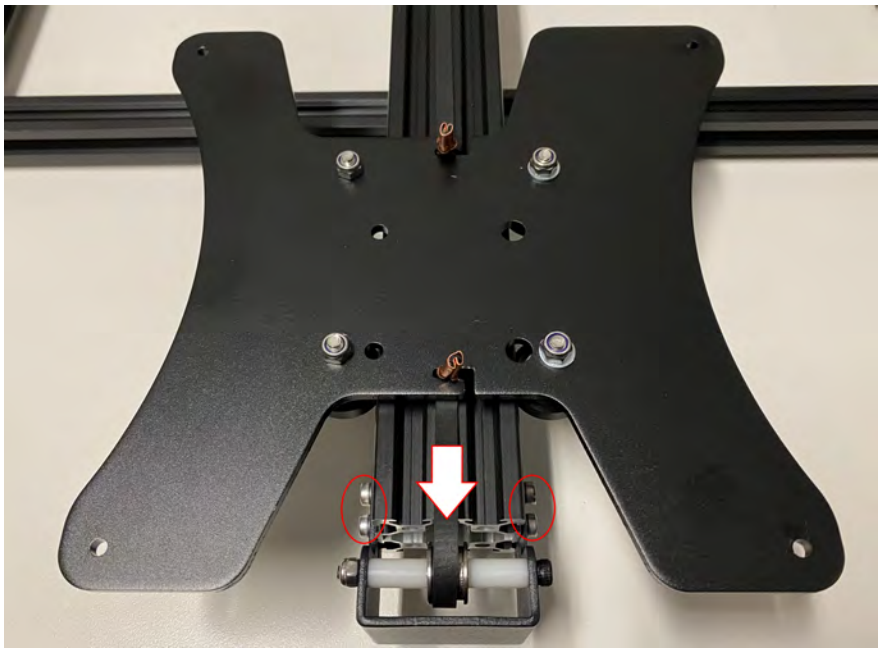


Figure A.36: **Y-Axis Belt Tensioner re-attachment.**

Remove the magnetic bed from the Heated Bed and remove the four screws by cutting holes around the screw heads.

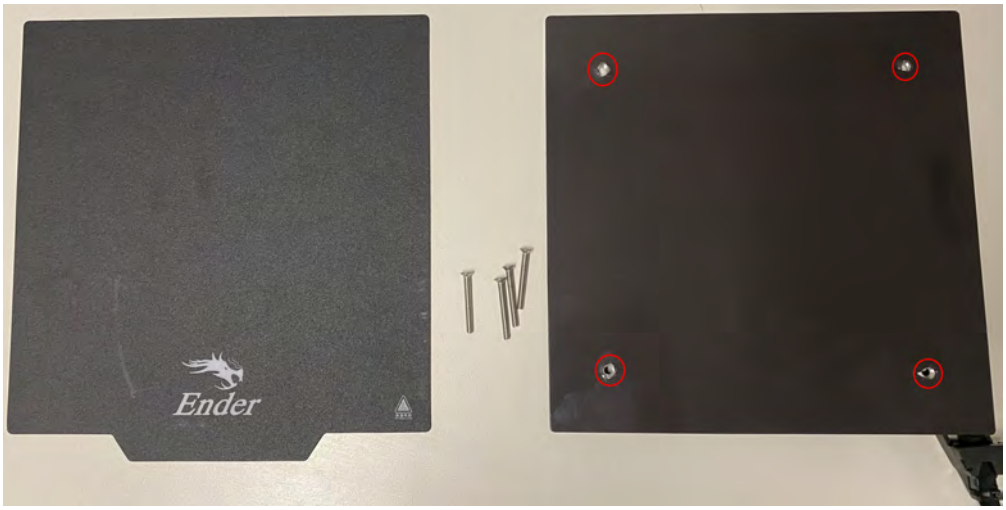


Figure A.37: **Heated Bed screw removal.**

Insert the four screws into the top face of the Ender Xtender Platform.

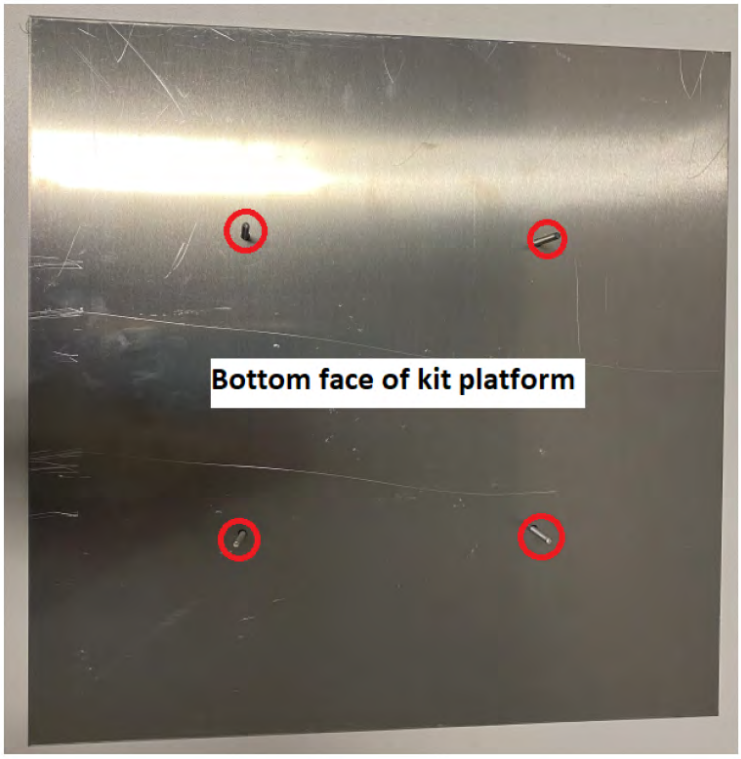


Figure A.38: **Heated Bed screw placement onto Ender Xtender Platform.**

Place the Heated Bed onto the Ender Xtender Platform.

Figure A.39: **Heated Bed placement over Ender Xtender Platform.**

Cut the ziptie and remove the plastic part from the wires.

Figure A.40: **Removing plastic part from Heated Bed wires.**

The Ender Xtender kit comes with four washers and four nuts.

Figure A.41: **Ender Xtender Platform nuts and washers.**

Place the washers on the screws then tighten the nut around the screws. This attaches the Heated Bed to the Ender Xtender Platform. Tape the remaining wires to the bottom of the Ender Xtender Platform and replace the four springs from A.13.

Figure A.42: **Heated Bed attachment to Ender Xtender Platform.**

Place the Ender Xtender Platform onto the Y-Axis Carriage being careful not to lose the springs. Attach the knobs from A.13 and tighten each knob until they are no longer loose. The platform is leveled with these knobs.

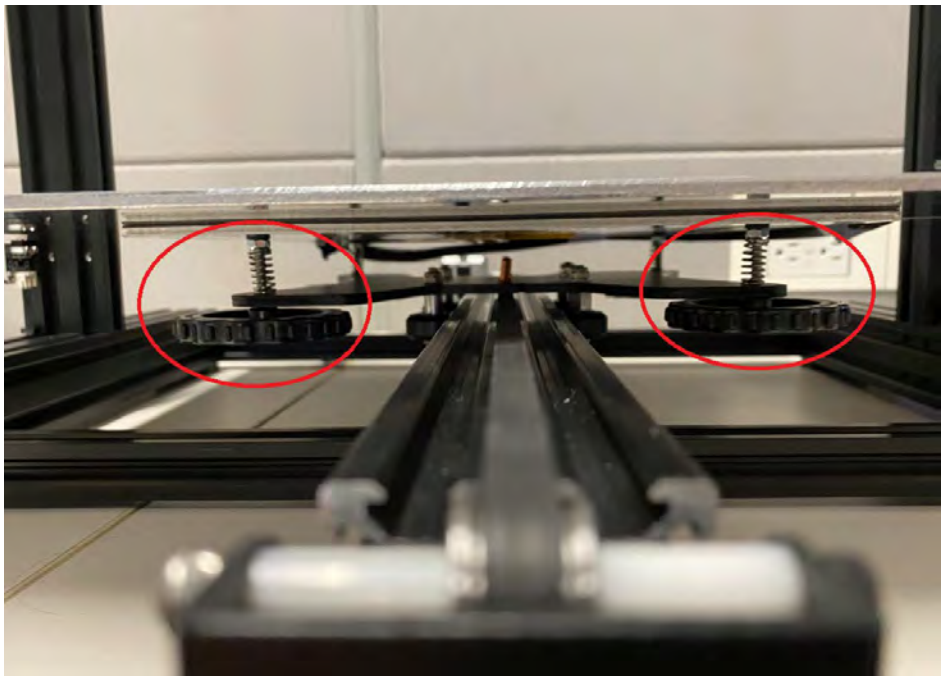


Figure A.43: **Ender Xtender Platform attachment.**

Gather the X-Axis Stepper Motor Assembly, the Z-Axis Roller and four M4x16 screws from the Ender 3 Pro supplies. Also grab the X-Axis Extrusion from the Ender Xtender kit.

Figure A.44: **X-Axis Components.**

Attach the Z-Axis Roller to the right side of the X-Axis Extrusion using two of the M4x16 screws.

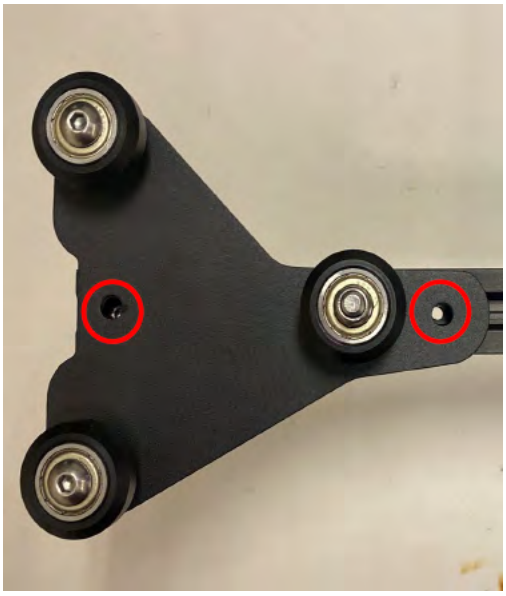


Figure A.45: **Z-Axis Roller attachment.**

Slide the Syringe Pump onto the X-Axis Extrusion. Feed the two wires from the limit switch through the Syringe Pump Holder as seen in the figure below.

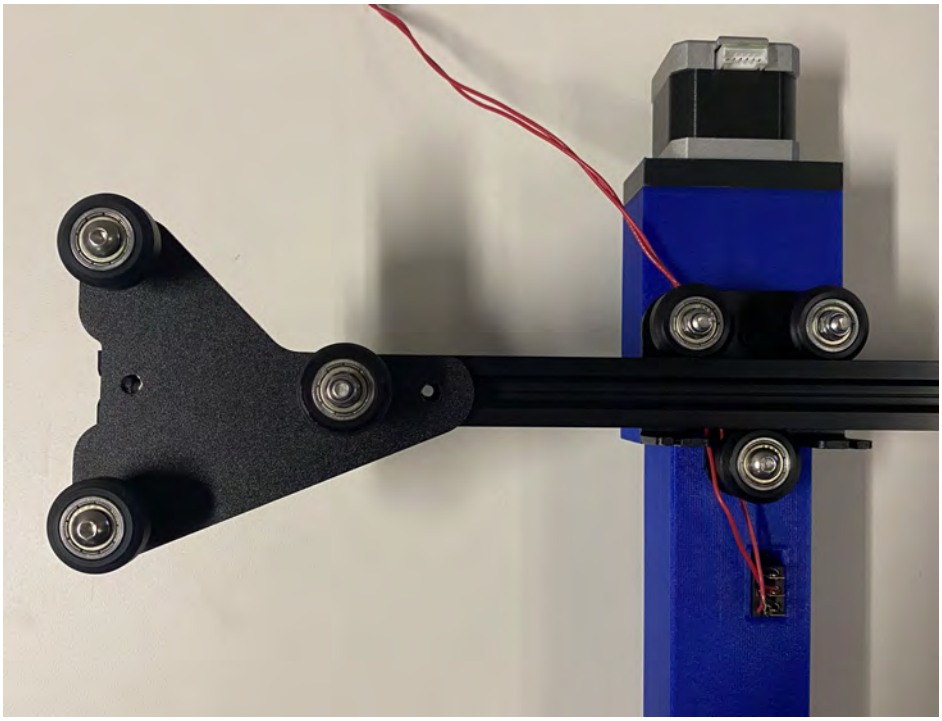


Figure A.46: **Pump attachment to X-Axis Extrusion.**

Attach the X-Axis Stepper Motor Assembly to the left side of the X-Axis Extrusion using two of the M4x16 screws.

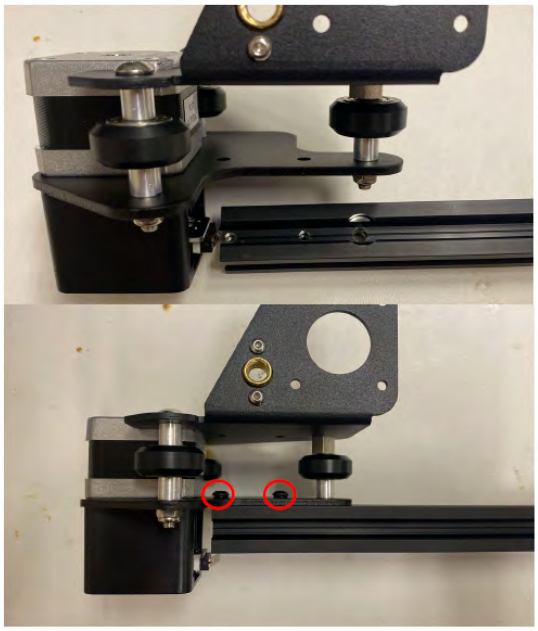


Figure A.47: **X-Axis Stepper Motor Assembly attachment.**

The result should like this:

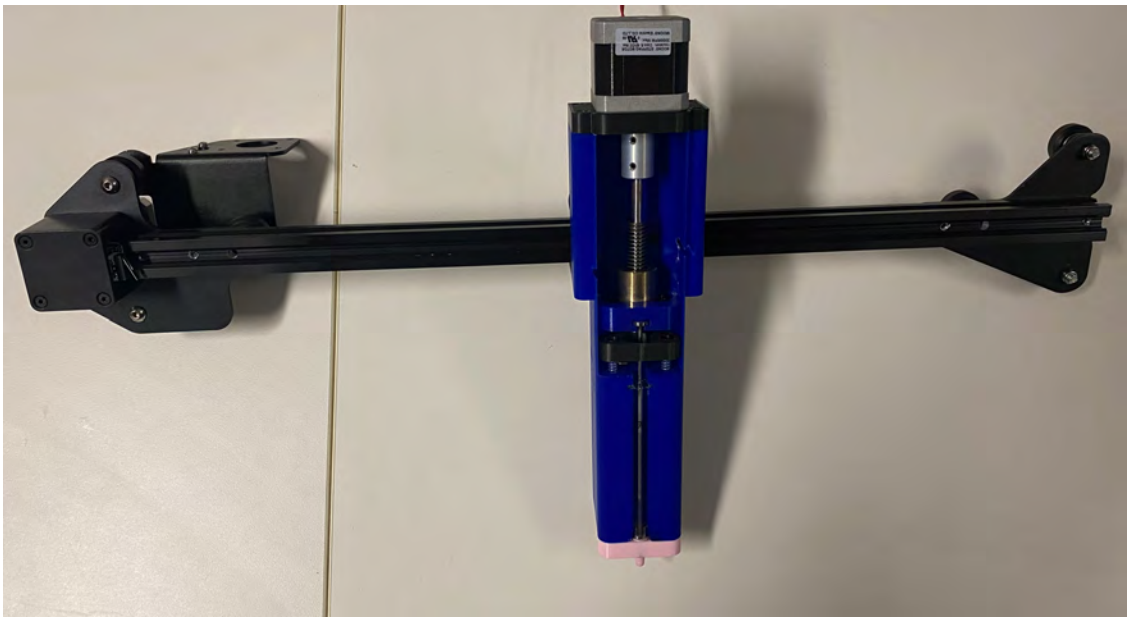


Figure A.48: **X-Axis Components assembled.**

Line up the rollers on each side with the two Vertical Extrusions to attach the X-Axis components to the frame.

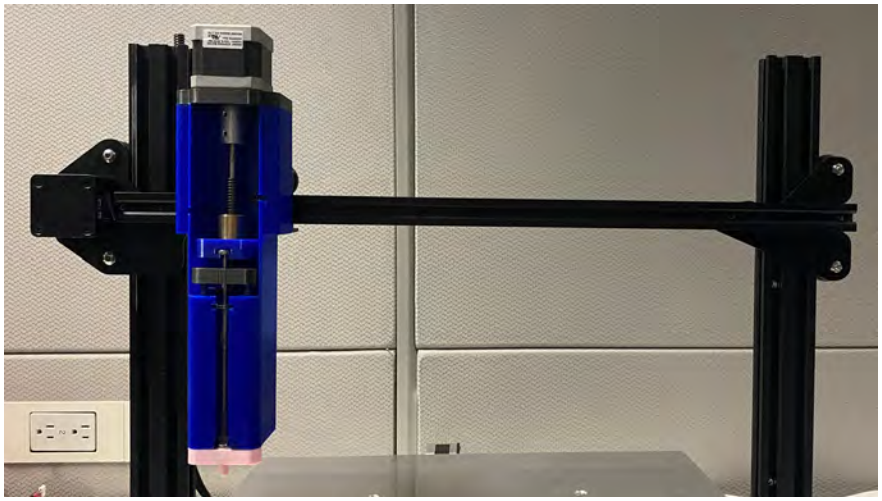


Figure A.49: **X-Axis components attached.**

Attach the Top Extrusion to the frame by using the four M5x25 screws provided with the Ender 3 Pro.

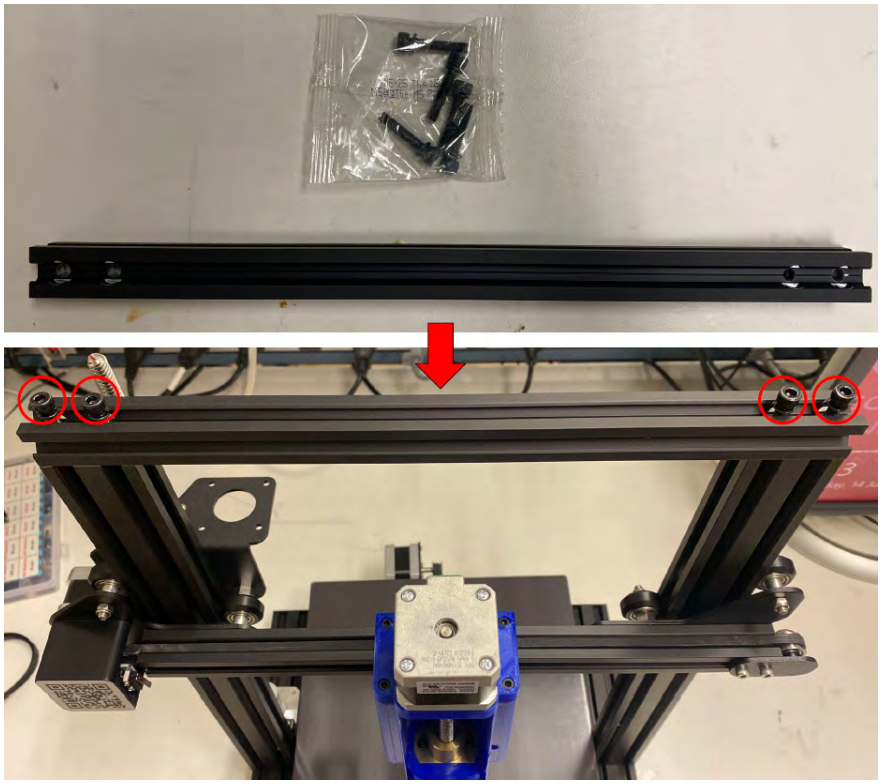


Figure A.50: **Top Extrusion attachment.**

Disassemble the Z-Axis Limit Switch that comes with the Ender 3 Pro by unscrews the two screws holding the switch and the two screws and T-nuts. Attach the switch to the new Z-Axis Limit Switch Holder using the two screws. Last, Attach the screws and T-nuts.

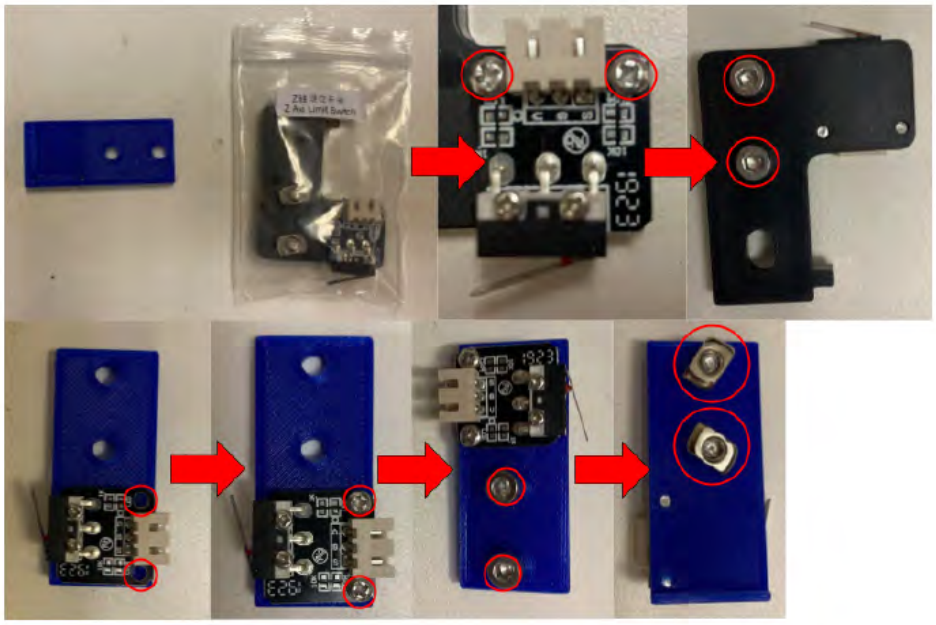


Figure A.51: **Z-Axis Switch assembly.**

Attach the Z-Axis Limit Switch to the back left of the Top Extrusion. Ensure it is flush with the Top Extrusion then tighten the T-nut screws.

Last, assemble the pieces of the BioCloneBot platform together and place them over the platform.

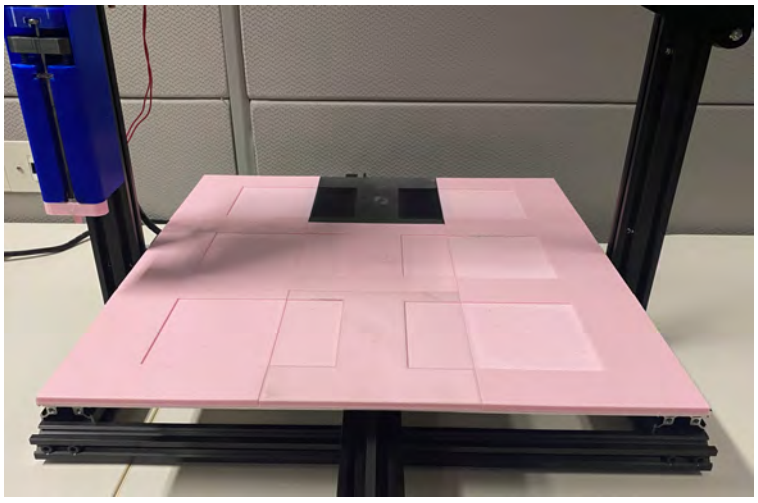


Figure A.52: **Platform attachment.**

Now the BioCloneBot frame and pump are fully assembled. The next section will cover the electronics.

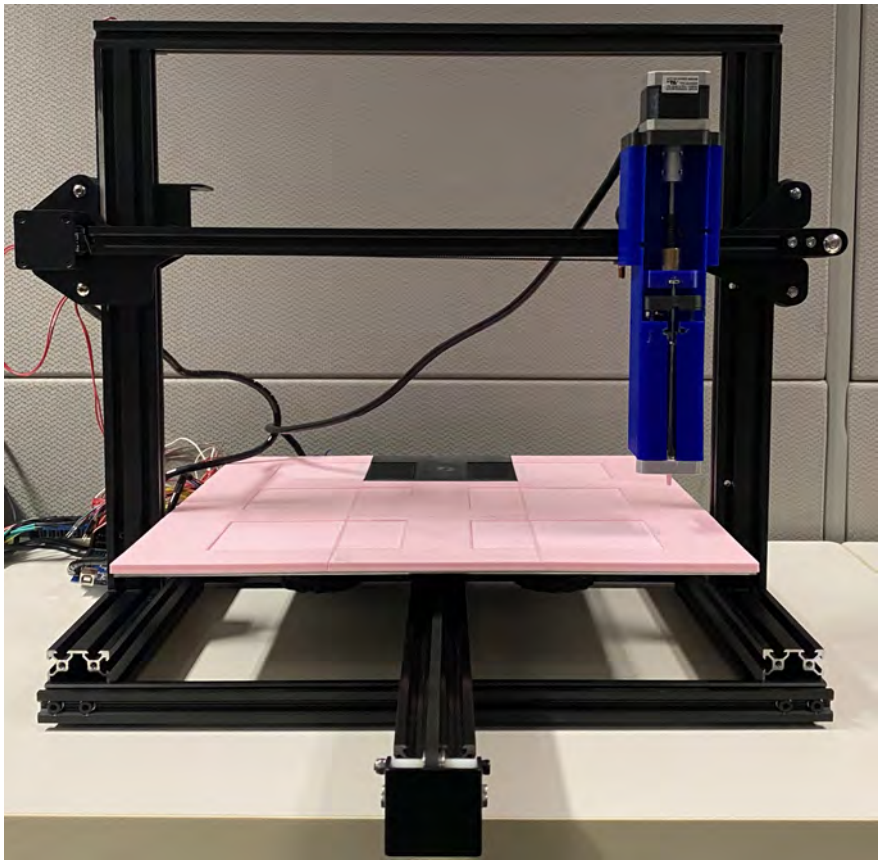


Figure A.53: **BioCloneBot frame and pump fully assembled.**

A.3 Electronics Assembly

All of the following parts are mentioned can be found in the bill of materials in Appendix C. Before beginning, ensure the power supply is **NOT PLUGGED IN** while cutting or soldering wires! In the following steps, only cut the end of the wire from the 24-pin connector.

Figure A.54: **Always ensure the power supply is OFF when cutting or soldering the wires.**

To power the BioCloneBot, a modular ATX PC power supply is used. Modular power supplies come with only the bare minimum wires hardwired to the power supply with extra wires that can be attached to power added PC components. For this guide, only the 24-pin ATX connector will be used. Following figure A.56 below, identify the function of each pin of the 24-pin ATX connector.

Figure A.55: **450W modular ATX PC power supply.**

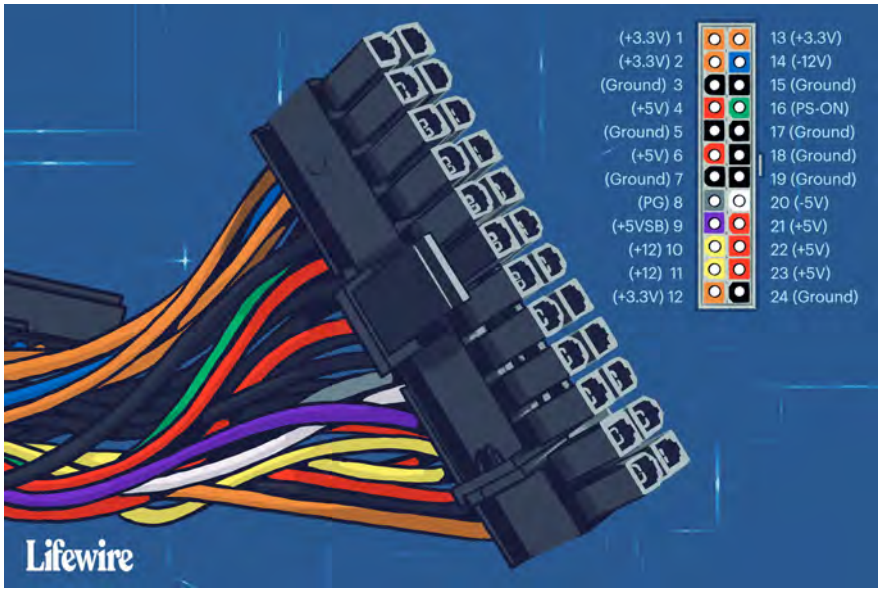


Figure A.56: **Standard 24-pin ATX power supply pin-out [49].**

Cut the PG wire and a Ground wire using wire cutters. Cut back some of PG wire sleeve then solder the exposed wire to one of the terminals of the on/off switch. Cut back some of Ground wire sleeve then solder the exposed wire to the other terminal of the on/off switch. Cover the exposed wire and terminals with electrical tape. When the power supply is plugged in and the switch on the power supply is turned on, this switch can be used to turn the power supply on or off. Use this as the emergency stop switch.

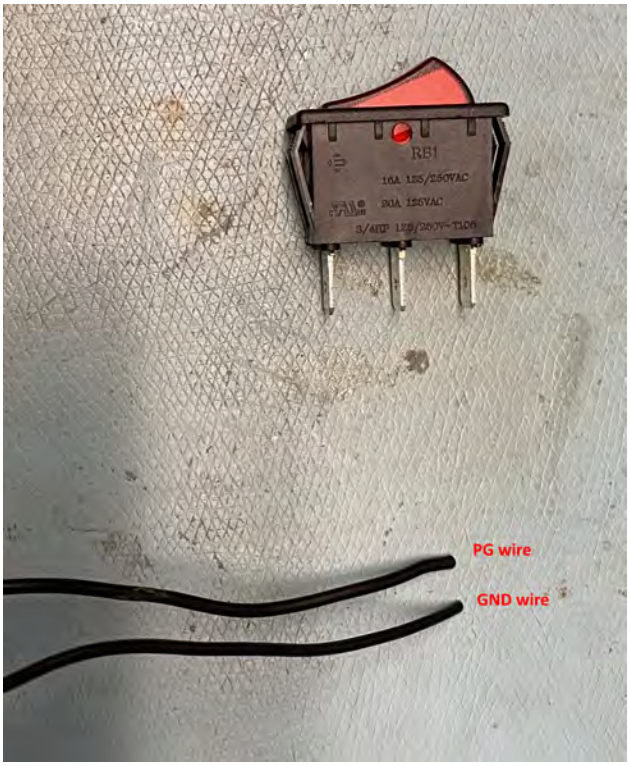
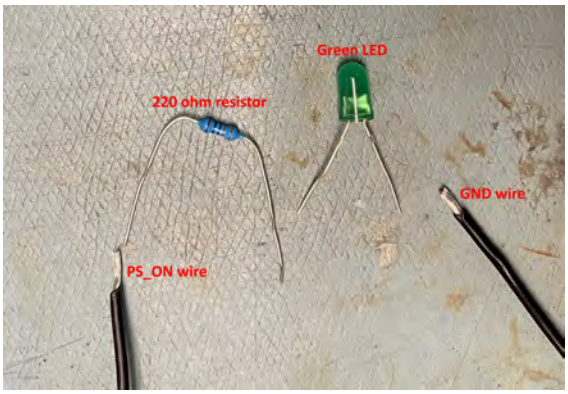
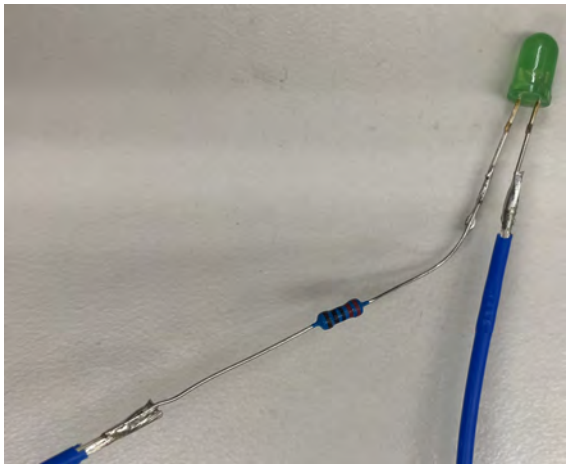


Figure A.57: **Power switch for emergency shut-off.**

Cut the PS_ON wire and one Ground wire using wire cutters. Cut back some of the PS_ON wire sleeve then solder one end of a 220Ω resistor to the exposed wire. Solder the positive lead of a green LED to the other end of the resistor. The positive lead is longer than the negative lead. Cut back some Ground wire sleeve then solder the negative terminal of the LED to the exposed wire. Cover the exposed wire, the resistor, and the terminals of the LED with electrical tape. **When the power supply is plugged in, the switch on the side of the power supply is turned on, and the soldered switch is on**, this LED will turn on.

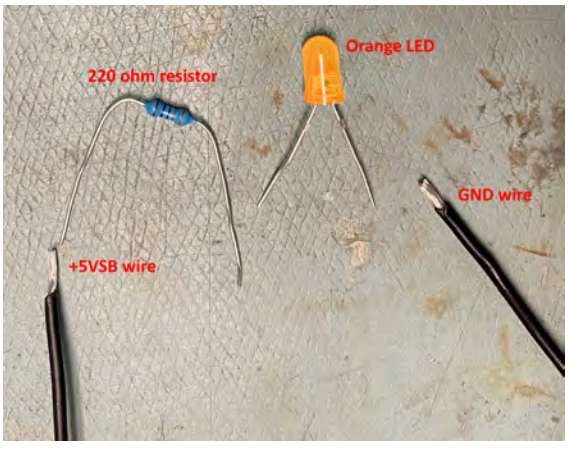


(a) **Preparing PS_ON LED.**

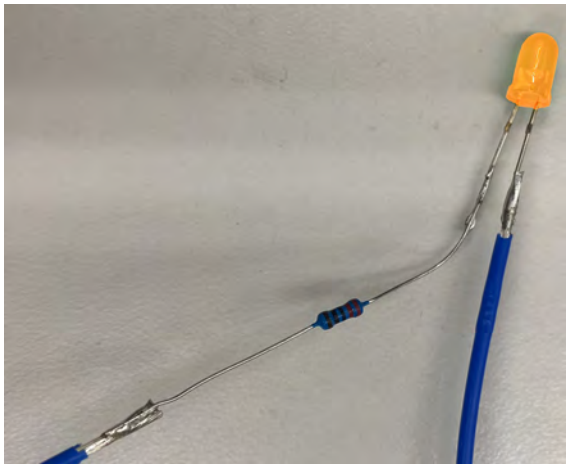


(b) **PS_ON LED soldered.**

Cut the +5VSB wire and a Ground wire using wire cutters. Cut back some of the +5VSM wire sleeve then solder one end of a 220Ω resistor to the exposed wire. Solder the positive lead of an orange LED to the other end of the resistor. The positive lead is longer than the negative lead. Cut back some Ground wire sleeve then solder the negative terminal of the LED to the exposed wire. Cover the exposed wire, the resistor, and the terminals of the LED with electrical tape. **When the power supply is plugged in, the switch on the side of the power supply is turned on**, this LED will turn on. This stays regardless of whether the soldered switch is on or off.



(a) **Preparing +5VSB LED.**



(b) **+5VSB LED soldered.**

The final LEDs look like this when everything is turned on. A table of the power states can be found in table A.1 below.

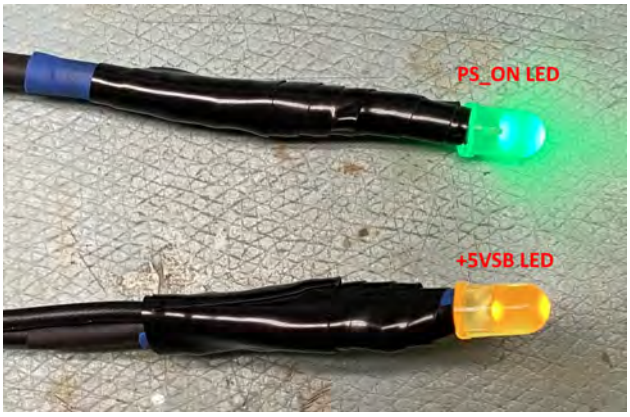


Figure A.60: **PS_ON and +5VSB LEDs turned on.**

PSU Cable	PSU Switch	Soldered Switch State	PS_ON LED State	+5VSB LED State
Not plugged in	-	-	Off	Off
Plugged in	Off	Off	Off	Off
Plugged in	On	Off	Off	On
Plugged in	Off	On	Off	Off
Plugged in	On	On	On	On

Table A.1: **Power supply switch states.**

Cut the −12V and −5V wire and cover them using electrical tape. They are not needed.

Cut all of the 3.3V wires. Separate the wires into two bundles. It is OK if the two bundles are not equal. Cut back the wire sleeve on each wire of both bundles. Take all the wires of one bundle then twist the exposed part of each wire into one big wire. Place the big wire into one of the solderless crimp terminal connectors. Despite being solderless, apply solder to attach the bundle to the connector. Do this for both bundles. Apply electrical tape to all exposed wire and solder. However you see fit, label the wires so it is clear that these are the 3.3V wires. Attach each bundle to on side of the terminal block.

Cut all of the 5.0V wires. Separate the wires into two bundles. It is OK if the two bundles are not equal. Cut back the wire sleeve on each wire of both bundles. Take all the wires of one bundle then twist the exposed part of each wire into one big wire. Place the big wire into one of the solderless crimp terminal connectors. Despite being solderless, apply solder to attach the bundle to the connector. Do this for both bundles. Apply electrical tape to all exposed wire and solder. However you see fit, label the wires so it is clear that these are

the 5.0V wires. Attach each bundle to on side of the terminal block.

Cut all of the 12.0V wires. Separate the wires into two bundles. It is OK if the two bundles are not equal. Cut back the wire sleeve on each wire of both bundles. Take all the wires of one bundle then twist the exposed part of each wire into one big wire. Place the big wire into one of the solderless crimp terminal connectors. Despite being solderless, apply solder to attach the bundle to the connector. Do this for both bundles. Apply electrical tape to all exposed wire and solder. However you see fit, label the wires so it is clear that these are the 12.0V wires. Attach each bundle to on side of the terminal block.

Cut all of the remaining Ground wires. Separate the wires into three bundles. It is OK if the last bundles are not equal to the other two. Cut back the wire sleeve on each wire of the bundles. Take all the wires of one bundle then twist the exposed part of each wire into one big wire. Place the big wire into one of the solderless crimp terminal connectors. Despite being solderless, apply solder to attach the bundle to the connector. Do this for all three bundles. Apply electrical tape to all exposed wire and solder. However you see fit, label the wires so it is clear that these are the Ground wires. Attach each bundle to on side of the terminal block.

Next we will make power wires for the motor drivers. Cut four wires of one color and four wires of another color that are long enough to reach from the terminal block to the breadboard. On one end of each wire, solder a terminal connector. On the other end, crimp a single male molex pin. Using the terminal connectors, attach two of the wires to one of the 12.0V lines on the terminal block. Do the same for the other 12.0V line. Repeat this using the second colored wires for the Ground lines.

The Ender Xtender kit comes with X, Y, Z, and E wires for the motors and limit switches (there is no E limit switch - for this we use the pump limit switch). Go ahead and cut the end of each wire that does not fit into the motor/limit switch. Crimp a 4-pin molex connector to the end of the stepper motor wires and a 2-pin molex connector to the end of the limit switch wires.

The circuitry can be wire by following this wiring diagram:

Appendix B

Software User Manual

The assembly guide, software, guide, BOM, and code base can be accessible through the following GitHub repo: BioCloneBot Github.

B.1 Prerequisites

Before you can modify the source code, you will need to install the following software:

Front-End

Visual Studio 2022 with .NET SDK 6.0+ Visual Studio 2022 Community Edition – Download Latest Free Version (microsoft.com)

If .NET cannot be found as in the error below:

Figure B.1: **.NET SDK cannot be found.**

Try the following fix:

c# - Microsoft Visual Studio 2019: The project file cannot be opened. Unable to locate the .NET SDK -

Stack Overflow

Firmware: Arduino IDE

Software — Arduino

B.2 Preparation

Before running experiments, it is important to ensure the following steps have been performed

- The red switch on the side of the power supply is set to 115V
- The BioCloneBot power supply is plugged in and turned on
- The Arduino Mega is connected to your computer using the provided USB cable
- You have uploaded the latest version of the BioCloneBot firmware to the Arduino
- The device has been calibrated (steps follow in the following section)

To home the BioCloneBot, remove everything from the platform and remove any tip attached to the syringe pump. Once the platform is clear, select the "Home Device" button underneath the Protocol Queue. The device will now home the x, y, z, and pump axes in order.

B.2.1 Calibration

Before running any experiments, it is crucial to calibrate the BioCloneBot. If you do not calibrate the device, it will miss the target during the experiments.

Here are the major calibration positions:

(1) The (x,y) homed position

(2) The top left corner location for the labware slots on the platform

(3) The (x,y) location for the center of the top left reservoir or tip

(4) The distance between the center of the reservoirs or tips

(5) The max height in the z-axis

(6) The height of the labware

Each calibration position can be set by using the Manual Control Form of the BioCloneBot software. Open the Manual Control Form by going to Settings -¿ Manually move pump.

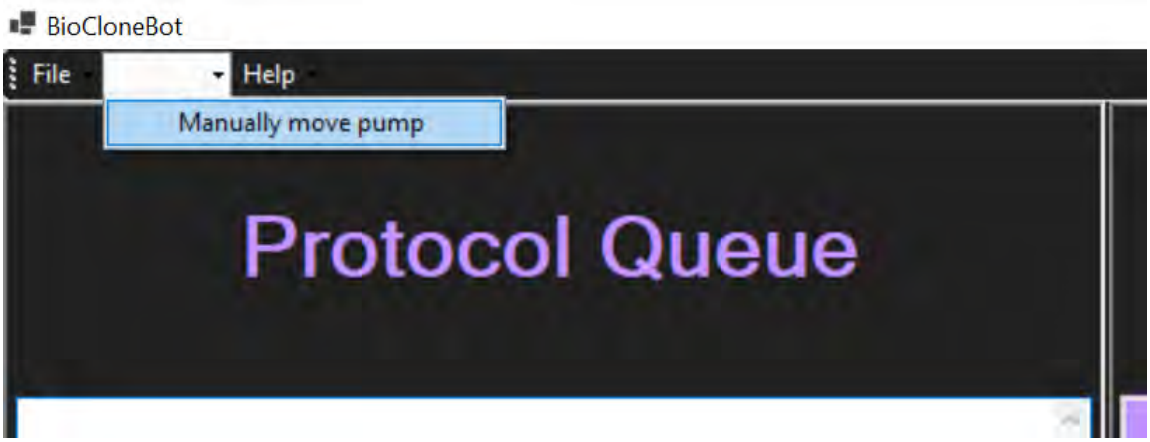


Figure B.2: **Opening the Manual Control Form.**

Carefully read the message pop-up then click OK.

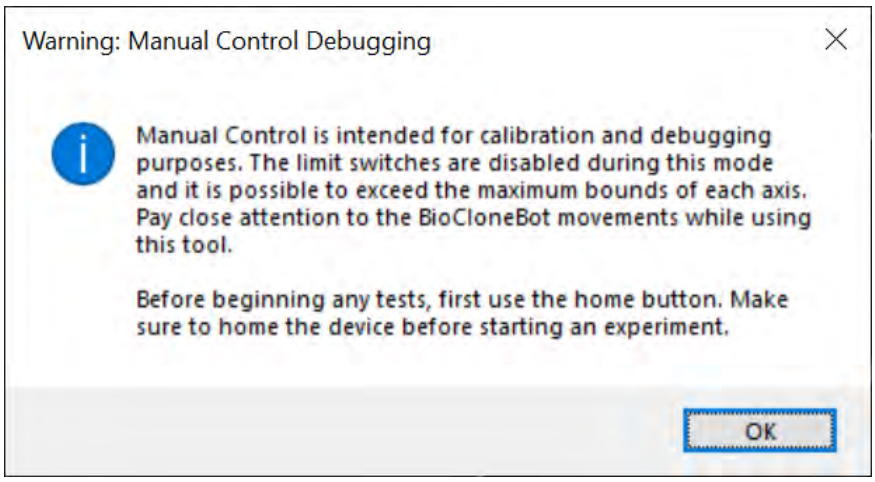


Figure B.3: **Manual Control Form warning pop-up.**

This will open the Manual Control Form

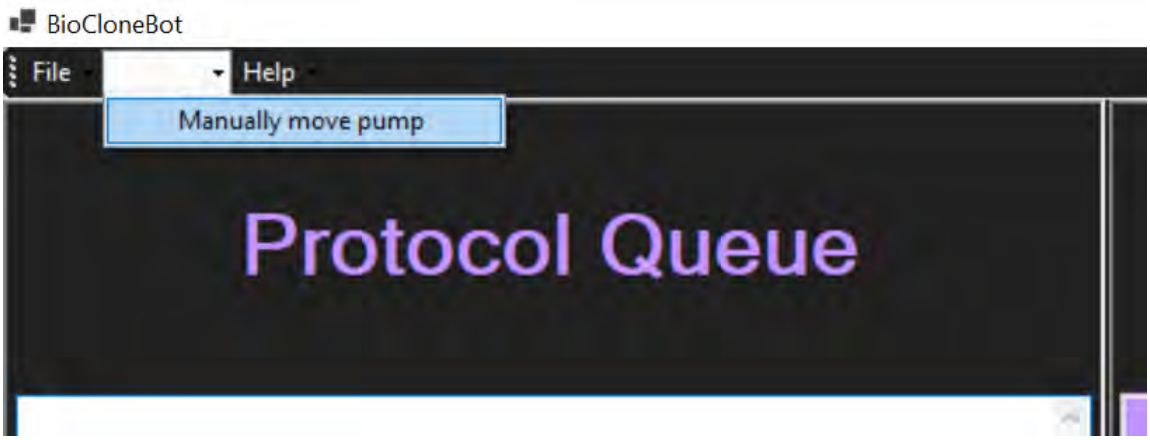


Figure B.4: **BioCloneBot Manual Control Form.**

To move a specific axis, enter a value into the "Travel Distance/Volume" text box, then press the corresponding button for the axis you would like to move. The + buttons move away from the limit switch of the axis and the - buttons move away from the limit switch of the axis. The aspirate and dispense buttons move the volume entered into the text box.

B.2.2 The (x,y) Homed Position

During the homing procedure, each axis starts by pressing the limit switch of that axis, then backing off until the switch is no longer pressed. Once finished, the center of the syringe pump tip will align with the bottom left of the platform. This refers to the (0, 0) position of the x-axis and the y-axis.

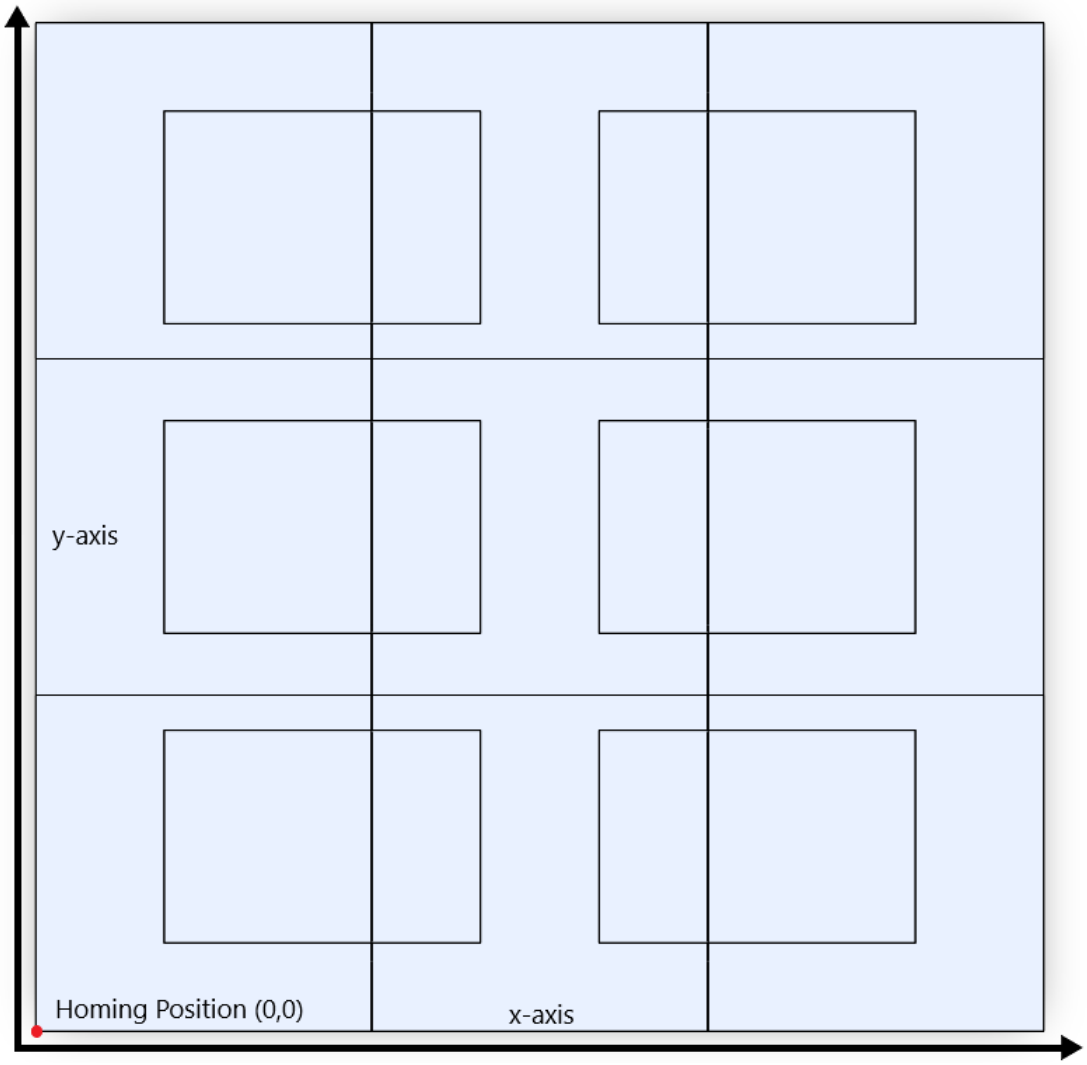


Figure B.5: **BioCloneBot platform with homed position in bottom-left corner.**

Press the home button and wait for the procedure to finish. Using the z+ button, move the pump until the pump is a couple cm above the platform. If the center of the syringe pump tip does not line up with the corner of the platform, open up the firmware file located inside the BioCloneBot code folder "BioCloneBot\Firmware\DeviceControllerV4\DeviceControllerV4.ino".

You can update the (x,y) homing location by modifying line 452.

```
movePump('1', '1', '0', 012.00, 033.00, 0.0);
//sets homing to 0 re-enabling the normal functionality of the limit switches as emergency stops
//and sets carriage location to (0.0, 0.0, 0.0) and syringe volume to 0
homing = 0;
```

Figure B.6: **Homing position inside of BioCloneBot firmware. Depending on the version of the code, the line is subject to change.**

012.00 refers to x-location and 033.00 refers to the y-location. Make sure to maintain the XXX.XX and YYY.YY format. The third number, 0.0 can be ignored as it refers to the point on the z-axis.

B.2.3 Top Left Corner of Labware Slots

The BioCloneBot uses the top left corner of each labware slot as the reference point when moving the pump to perform an operation. These locations can be seen below:

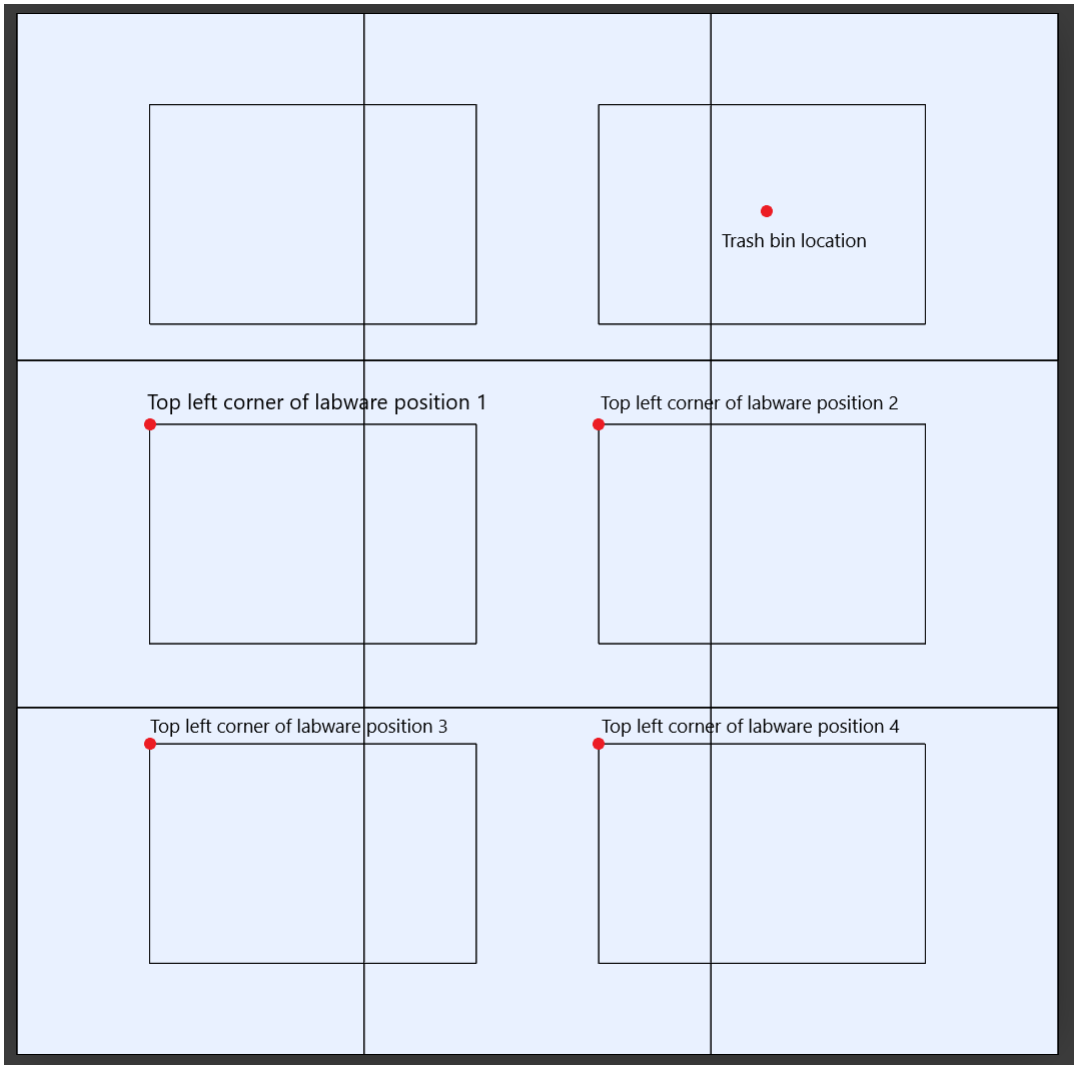


Figure B.7: **Top left reference corner for each labware slot on the BioCloneBot platform.**

These positions can be modified inside of the Platform.cs file located in "BioCloneBot\BioCloneBot\Platform.cs" The positions can be modified in lines in the AddLabware function of the Platform.cs class. topLeftCorner[0] refers to the x-location and topLeftCorner[1] refers to the y-location.

B.2.4 (x,y) of Top Left Reservoirs

The next position to calibrate is the distance from the top left corner of the labware slot to the center of the corresponding labware reservoir.

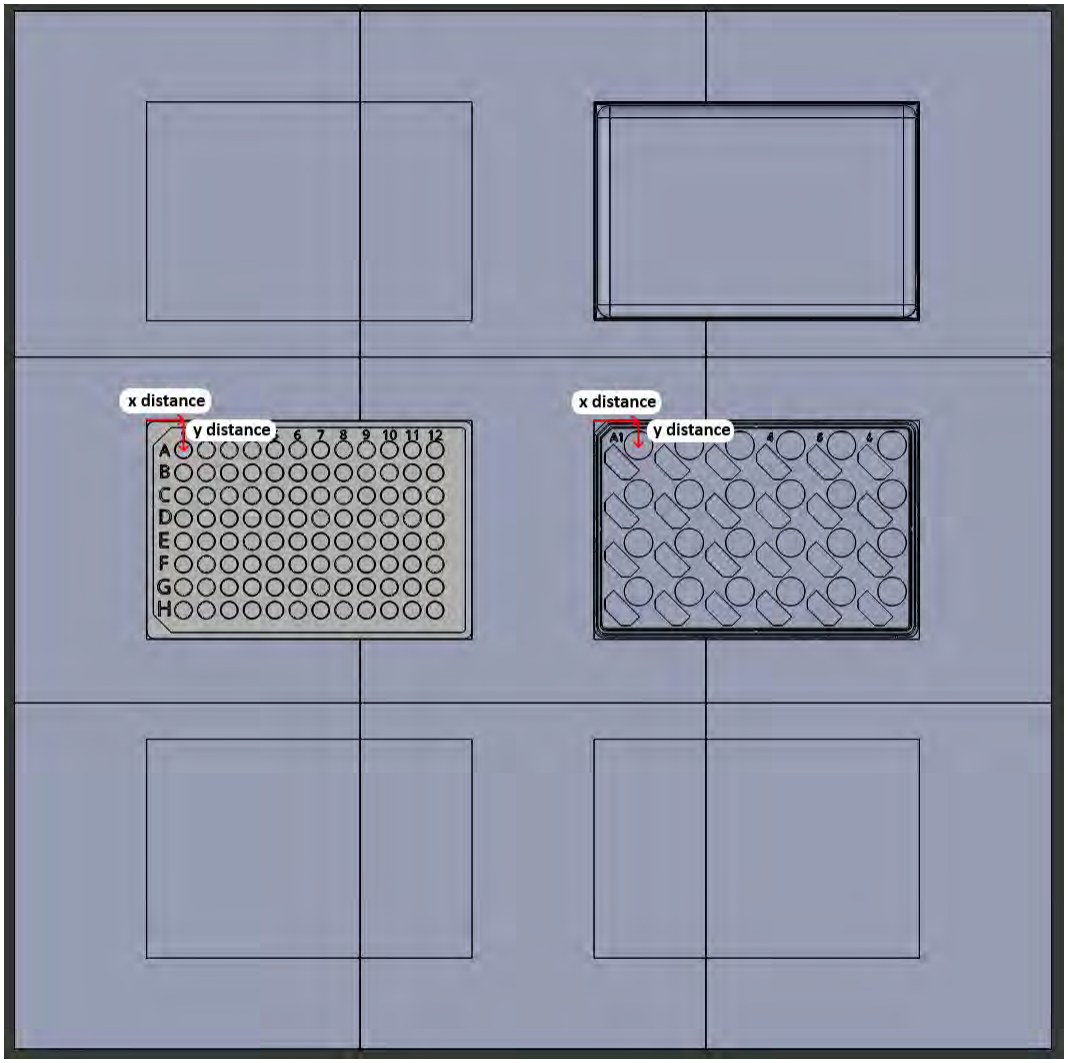


Figure B.8: **X and y distance from the top left corner of the labware position to the center of the top left reservoir.**

These can be be modified by updating the ”startLocation” variable inside of the Wellplate.cs, Tubestand.cs, and Tipbox.cs classes. The classes can be found inside the “BioCloneBot\BioCloneBot\” folder. The first value is the x-distance and the second value is the y-distance.

B.2.5 Reservoir Distances

Now that we have calibrated the top left corner of the labware locations and the start location of the first reservoir, we need to calibrate the distance between the reservoirs.

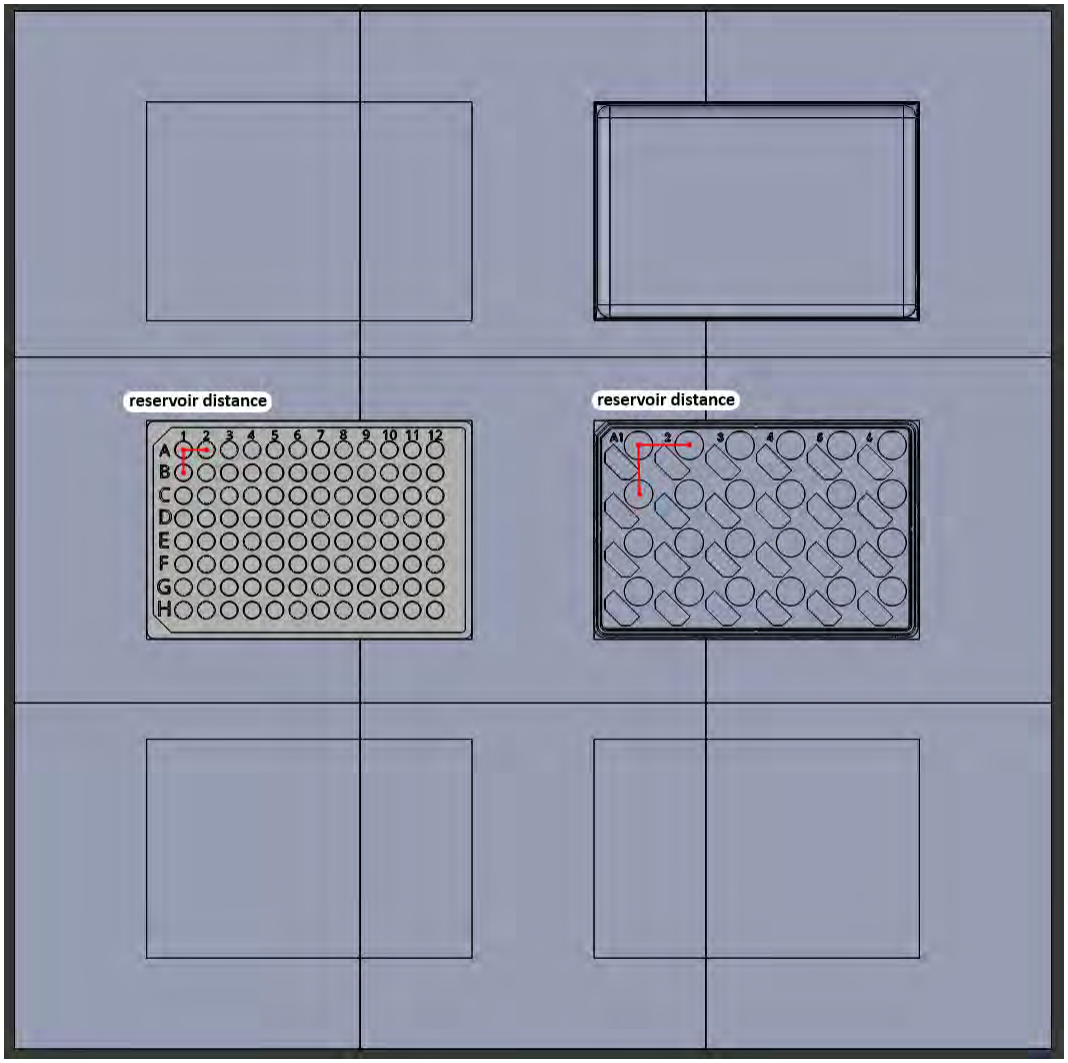


Figure B.9:

This can be updated inside of the class file for each labware: Wellplate.cs, Tubestand.cs, andTipbox.cs located in “BioCloneBot\BioCloneBot\”. Here are the variable names for each class:

”wellDistance” inside of Wellplate.cs ”tubeDistance” inside of Tubestand.cs ”tipDistance” inside of Tipbox.cs

B.2.6 Set Z-Max Value

The BioCloneBot uses Z-Max (the maximum travel distance in the z-axis) to help target labware during an operation. To calibrate Z-Max, home the device without a pipette tip attached.

Once homed, lower the pump until the tip adapter is touching the platform, but not pushing it down. This is

the Z-Max value. This can be updated inside the Platform class file located at “BioCloneBot\BioCloneBot\Platform.cs” inside of the Platform constructor function.

B.2.7 Height of Labware

The labware height refers to the target location of the pump depending on the labware to target. If you are trying to attach a tip, assume that there is no tip attached. If you are targeting a wellplate or tube reservoir, attach a pipette tip to the syringe pump by hand to account for the extra distance added by the tip. You will need to use the Manual Control Form to figure out these values. You will need to move the pump in each axis until it is in the desired location. The final value for the z-location is the value to input.

Pipette Tip

Move the pump until the tip adapter is located over the center of a pipette tip. Lower the pump until the adapter is inside the tip, but it is not snug. The tip attachment process automatically lowers the syringe pump after it is located inside of the tip to ensure a snug fit.

Wellplate or tubestand

Move the pump until the pipette tip is located over the center of the reservoir. Lower the pipette tip until it has touched the bottom of the reservoir then back up around 0.5 mm. This will ensure the pipette tip is not blocked when trying to aspirate or dispense.

The labware height can be updated inside of the class file for each labware: Wellplate.cs, Tubestand.cs, and Tipbox.cs located in “BioCloneBo\BioCloneBot\”. The third number in the ”dimensions” variable refers to the measured height.

B.3 Setting Up an Experiment

Now that the labware has been defined and initialized, we can start building our experiment. There are currently 6 operations available:

- Home Device: homes the x, y, z, and pump motors
- Get Tip: gets tip from a selected position on an available tip box
- Remove Tip: unloads tip into the trash bin
- Aspirate: aspirates a volume in μL into the pipette tip

- Dispense: dispenses a volume + 25μL to a destination reservoir
- Mix: mixes a volume a specified number of times in a destination well

Though the device should already be homed, it is best practice to start a protocol with aHome Device Operation. Select the Home Device Operation and note that it fills the protocol queue.

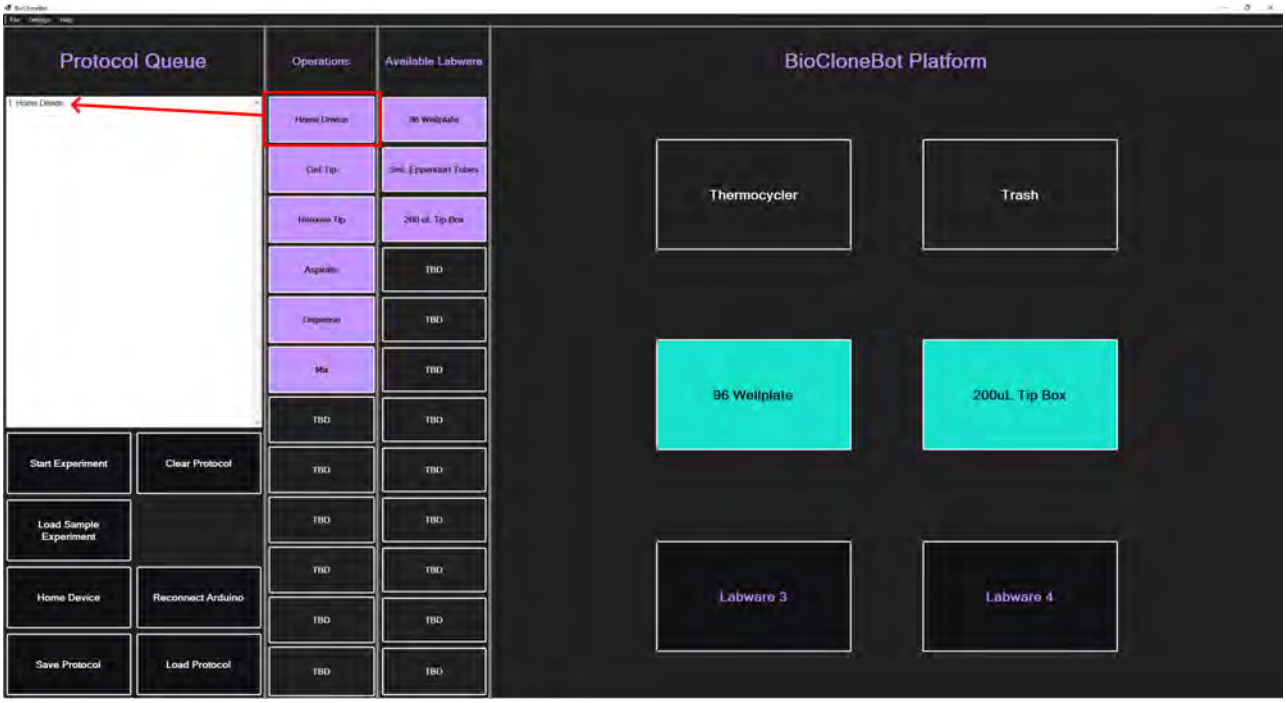


Figure B.10: **Add Home Device operation.**

Before we can aspirate or mix, we need to attach a pipette tip to the pump. Select the “Get Tip” operation then select the 200μL Tip Box on the platform.

Figure B.11: **Add Get Tip Operation.**

The Attach Tip Operation form will now open.

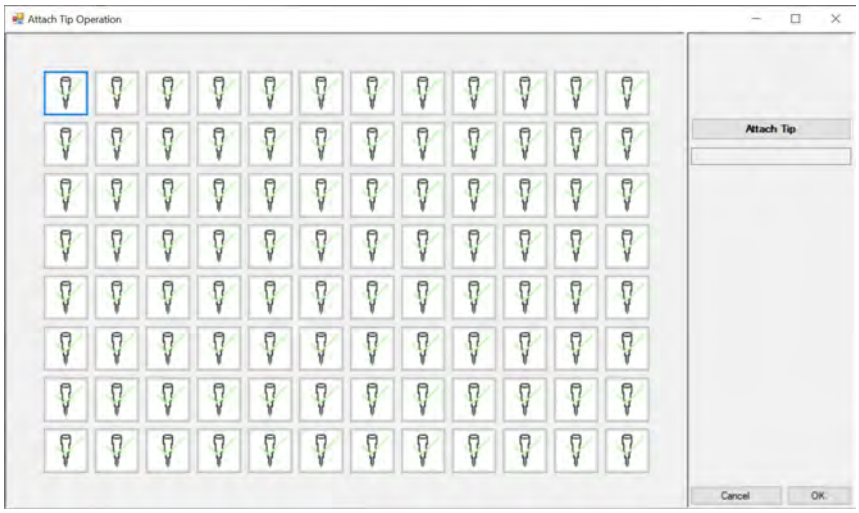


Figure B.12: **Get Tip Operation Form opened.**

Select the top left tip, then click the Attach Tip button.

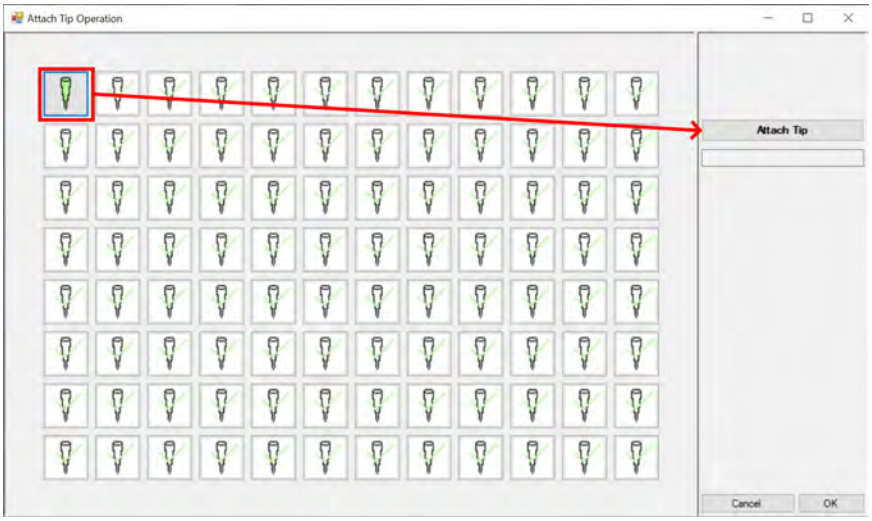


Figure B.13: **Select tip and click Attach Tip to attach the selected tip.**

Notice that the tip is no longer available for selection.

Figure B.14: **Tips are no longer available after being attached.**

Press OK to go back to the platform.

Now we can see the second step in the Protocol Queue “Get Tip from tipbox in position 2.” 5.

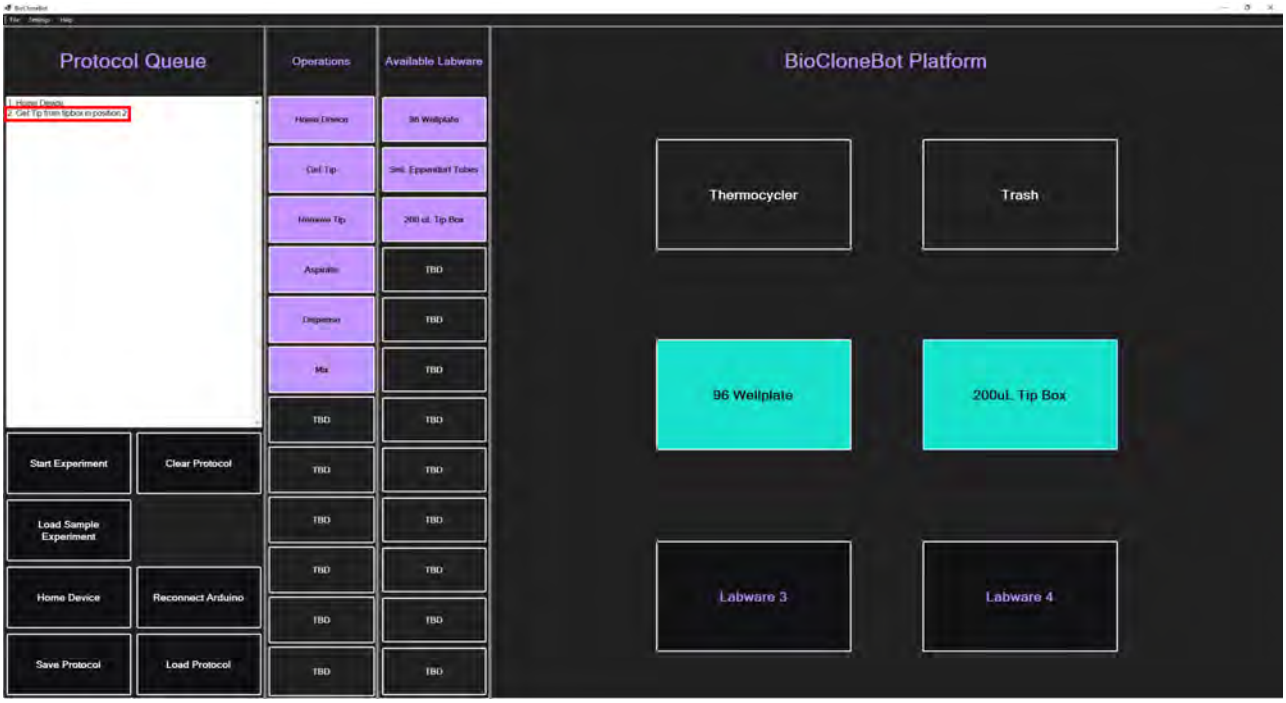


Figure B.15: **The Get Tip Operation is added to Protocol Queue after confirming.**

Next, select the Aspirate operation then select the 96 wellplate.

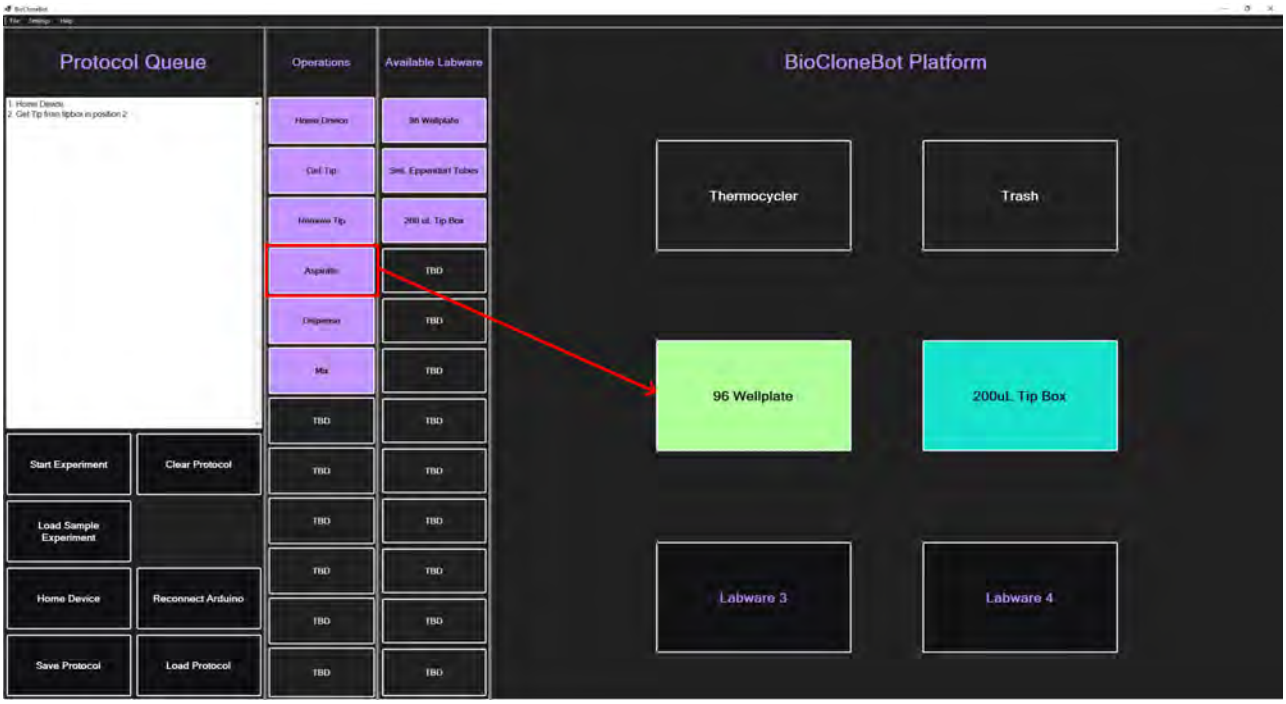


Figure B.16: **After clicking the Aspirate Operation button, the wellplate lights up.**

This will open the Aspiration Form.

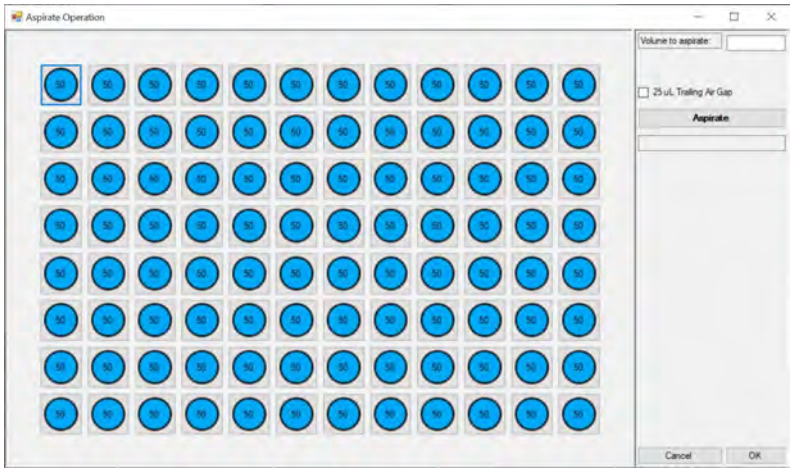


Figure B.17: **Aspiration Operation form.**

Select the top left well and enter in 50 into the Volume to aspirate text box, and click Aspirate.

Figure B.18: **Select the well, enter in 50μL, then click Aspirate to confirm the Operation.**

We can see that the operation was confirmed and that 50μL was removed from the well.

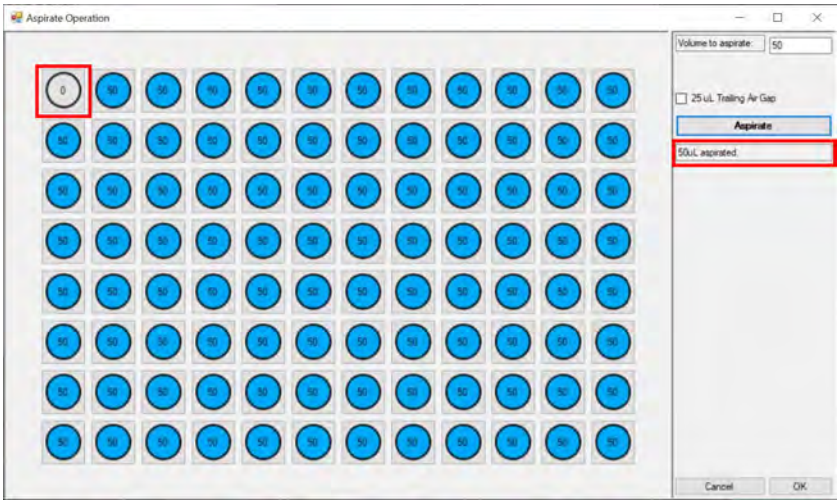


Figure B.19: **The well automatically updates to 0μL after confirming the operation.**

Note: You can use the 25μL Trailing Air Gap check box to aspirate 25.0μL of air before aspirating the designated volume from the well. This 25.0μL can then be used to simulate the double press action of a pipette where a larger volume is dispensed than picked up to guarantee all the liquid is ejected from the pipette tip. In this case, we will leave it blank.

Click OK and we will now see that the aspiration step has been added.

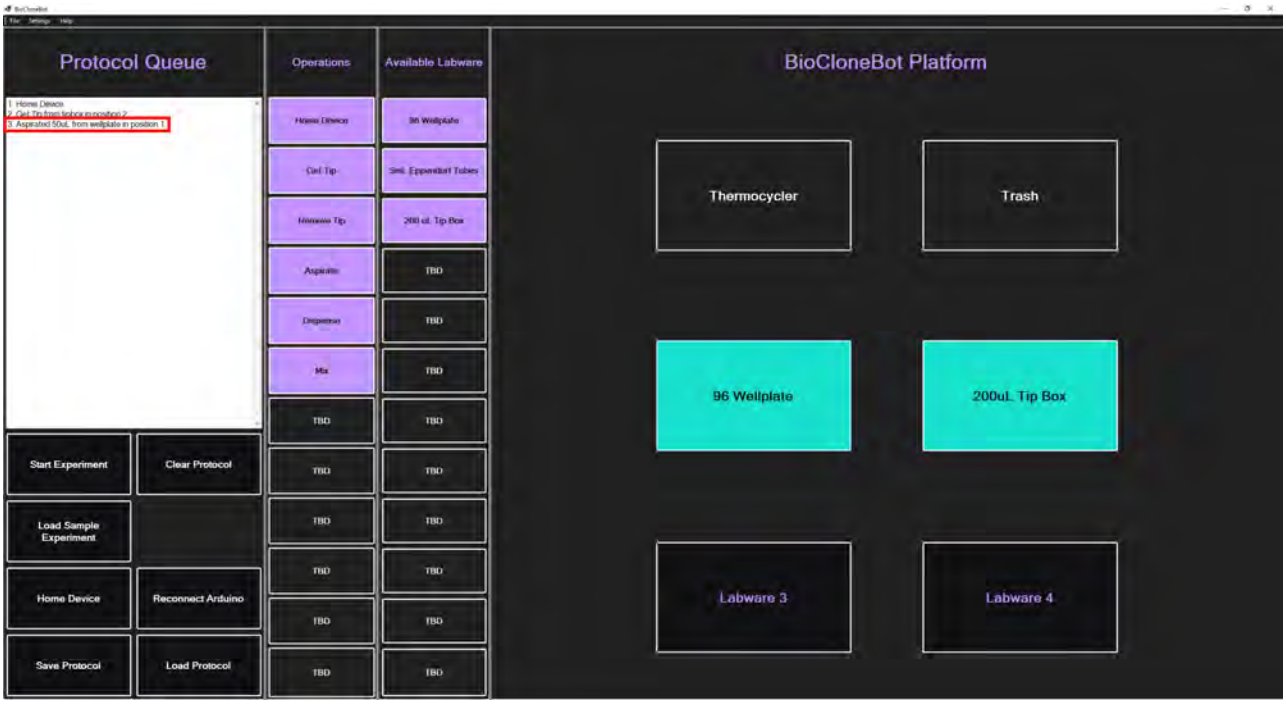


Figure B.20: **The Aspiration Operation was added to the Protocol Queue.**

Next we will dispense the 50μL. Select the Dispense operation then select the 96 wellplate.

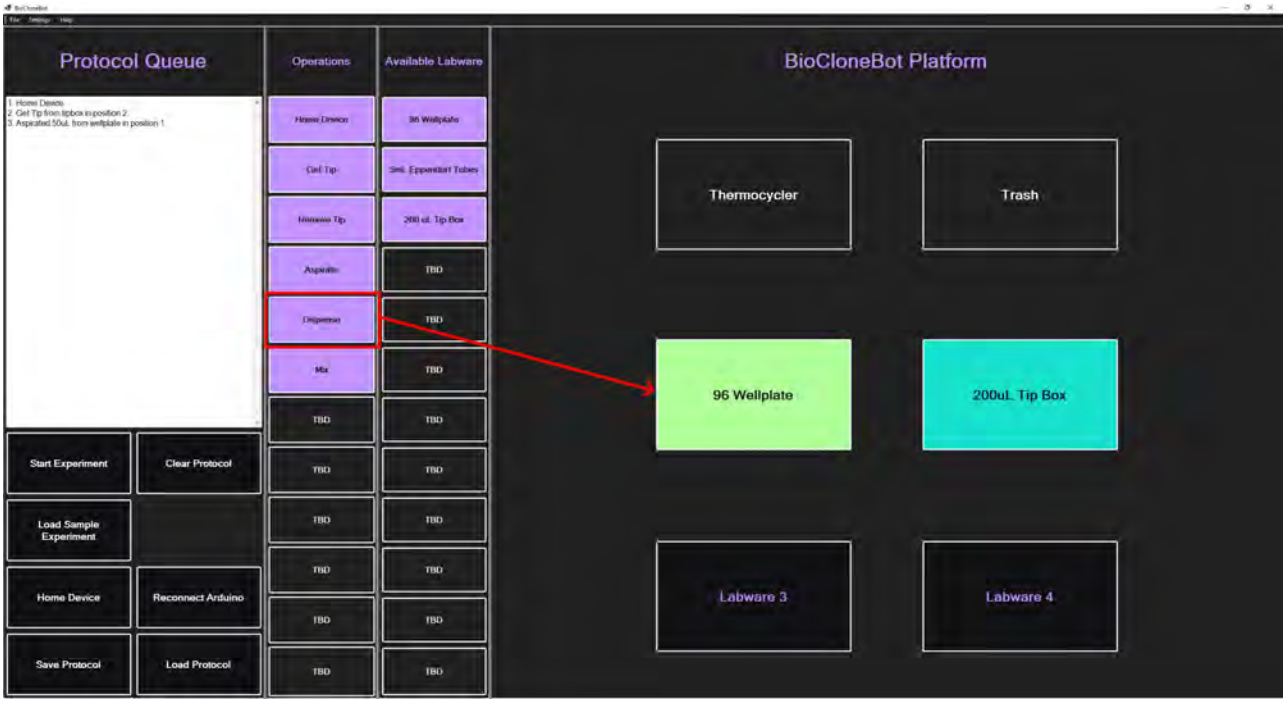


Figure B.21: **When selected the Dispense Operation, the wellplate lights up.**

This will open the dispense operation form.

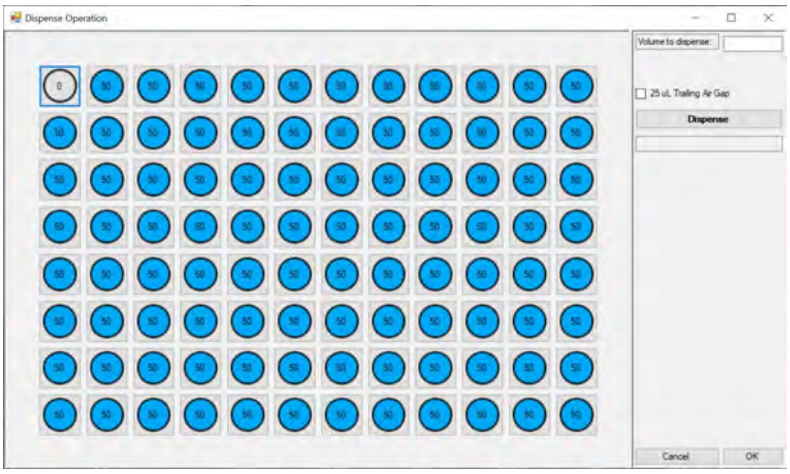


Figure B.22: **Dispense Operation Form.**

Select the top right well, enter 50 into the Volume to dispense text box, and click Dispense.

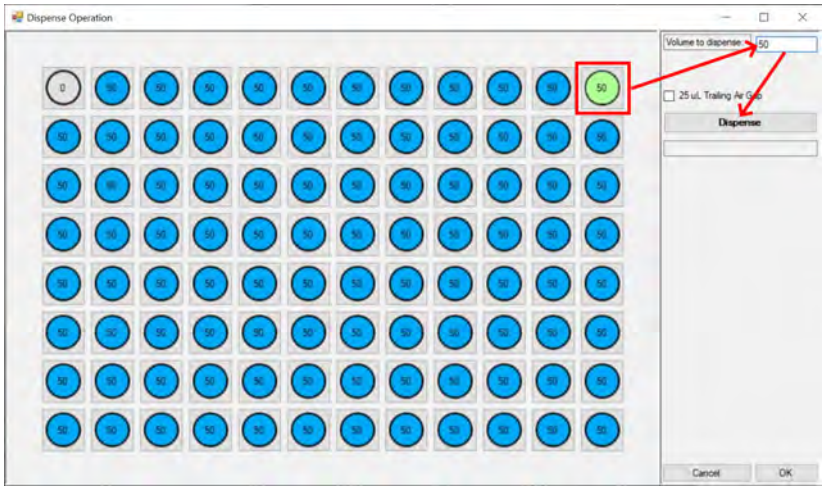


Figure B.23: **Select the well, enter in 50μl, then click dispense.**

Now we can see that 50μL was dispensed to the top right well.

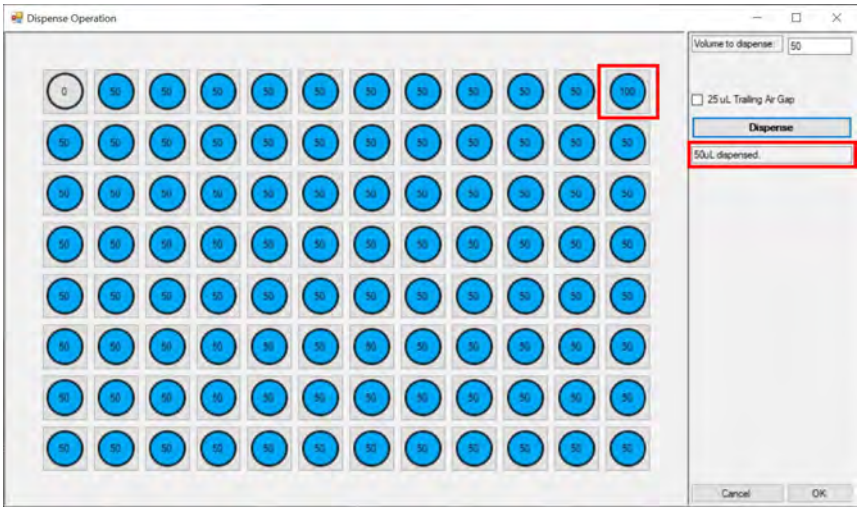


Figure B.24: **The well updates to 100μL after clicking Dispense.**

Click OK and we will see the dispense operation is added to the Operations Queue.

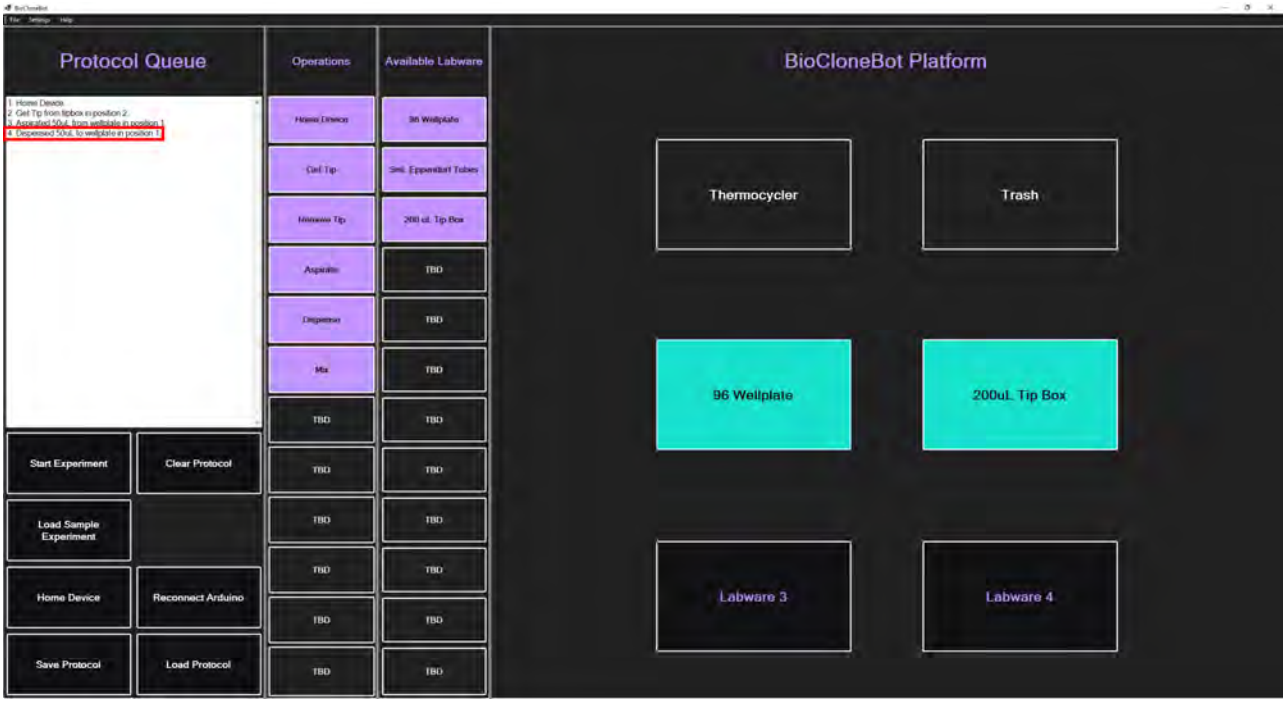


Figure B.25: **The Dispense Operation was added to the Protocol Queue.**

Next, select the Remove Tip operation. The software knows where the trash bin is so you do not need to select anything additional.

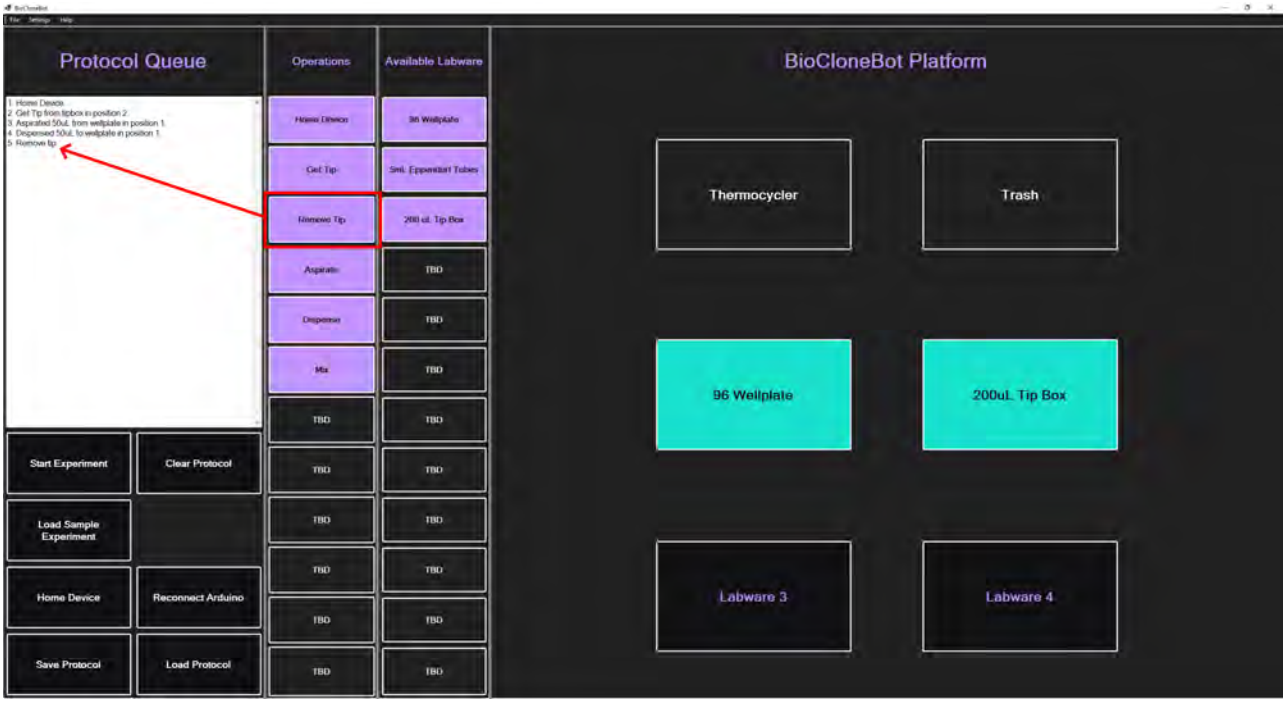


Figure B.26: **Pressing the Remove Tip Operation button adds a second Remove Tip to the Protocol Queue.**

Select the Get Tip operation and select the 200μL Tip Box.

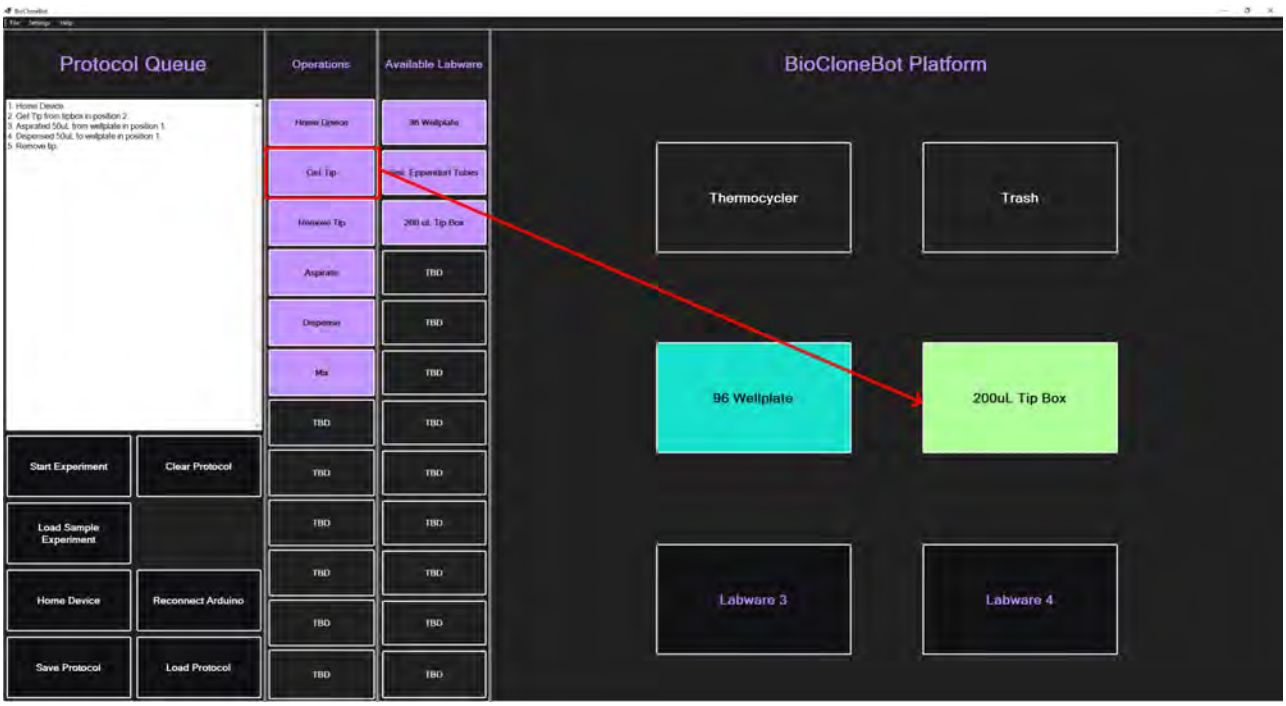


Figure B.27: **Select Get Tip to attach a fresh tip to the pump.**

Select the tip to the right of the used tip location and select Attach Tip.

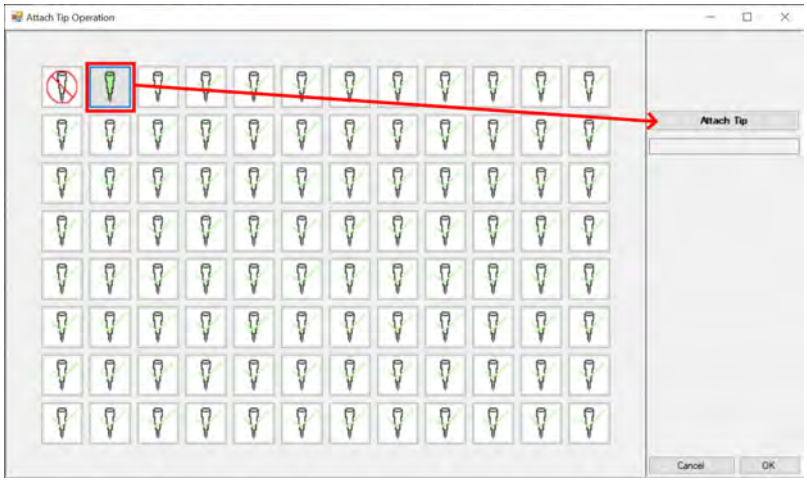


Figure B.28: **Select the second tip and click Attach Tip.**

We can see the second tip is removed and the new tip is attached.

Figure B.29: **The second tip is no longer available.**

Click OK to return back to the Control Form. We can see the get tip operation was successful.

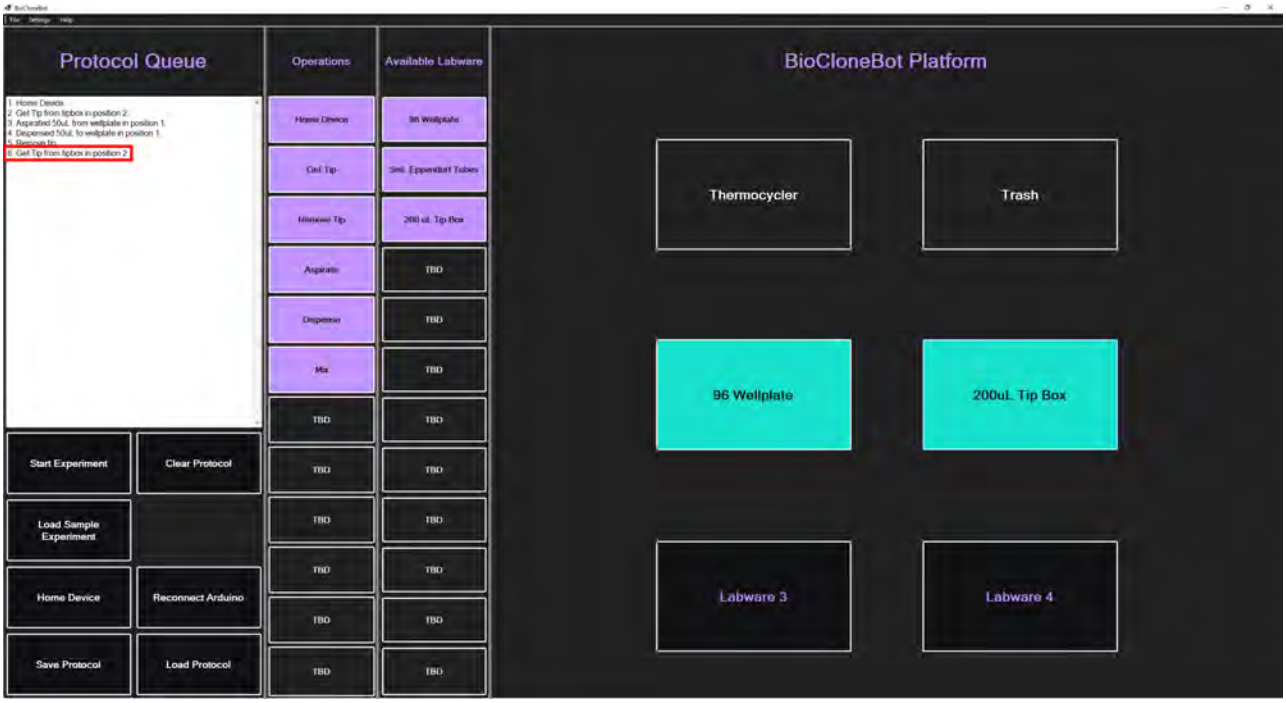


Figure B.30: **The second Get Tip Operation is added to the Protocol Queue.**

Next we will select the mix operation. Click Mix and select the 96 wellplate.

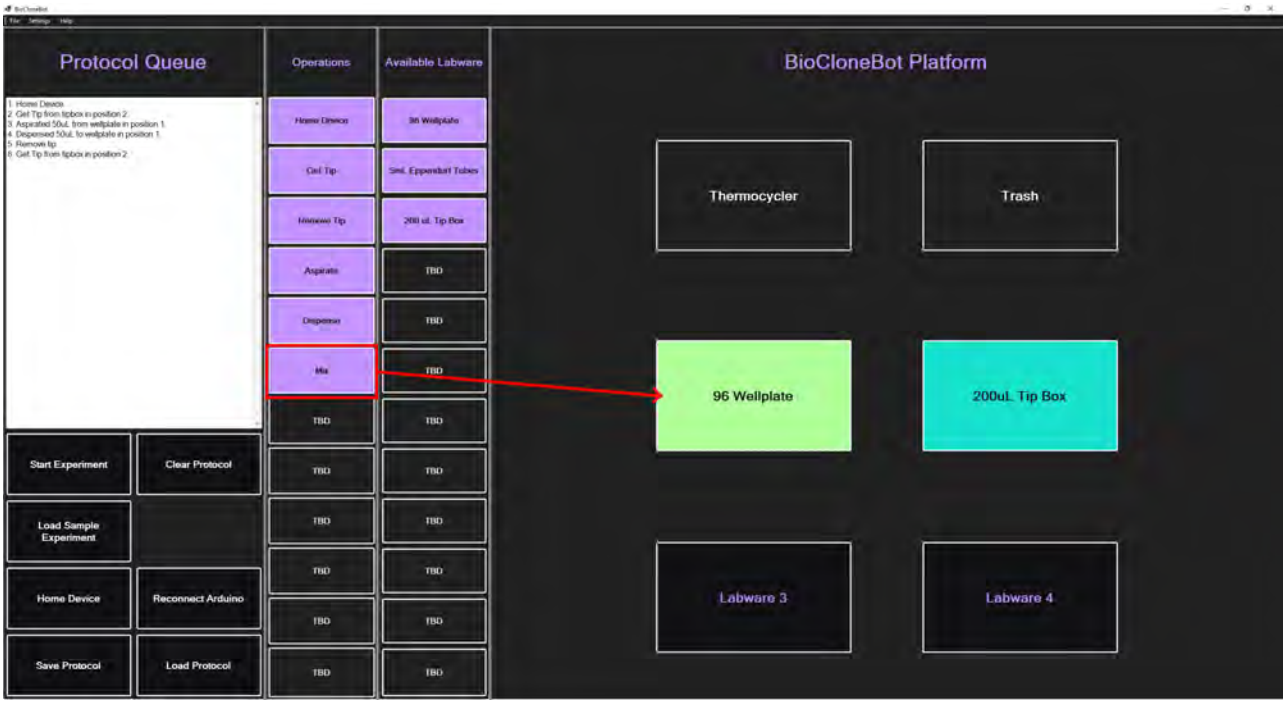


Figure B.31: **Selecting the Mix Operation highlights the 96 wellplate.**

The mix operation form will open.

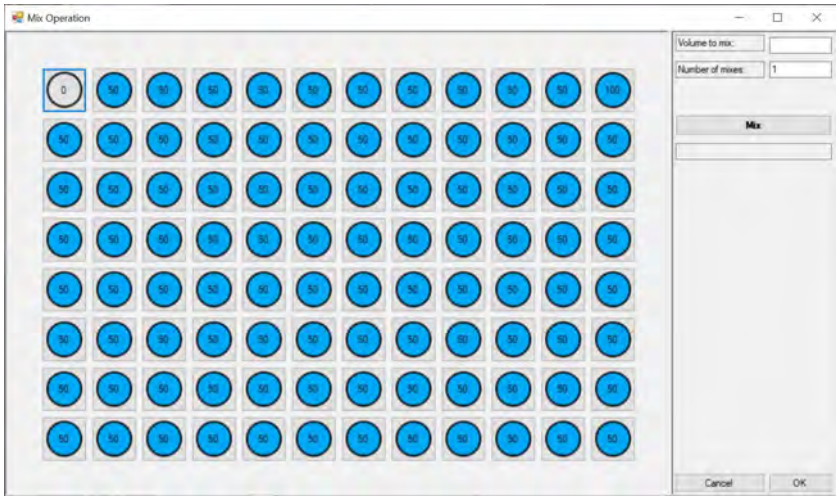


Figure B.32: **The Mix Operation Form.**

Select the top right well, enter in 25 into the Volume to mix text box, enter 5 into the number of mixes text box, and click Mix.

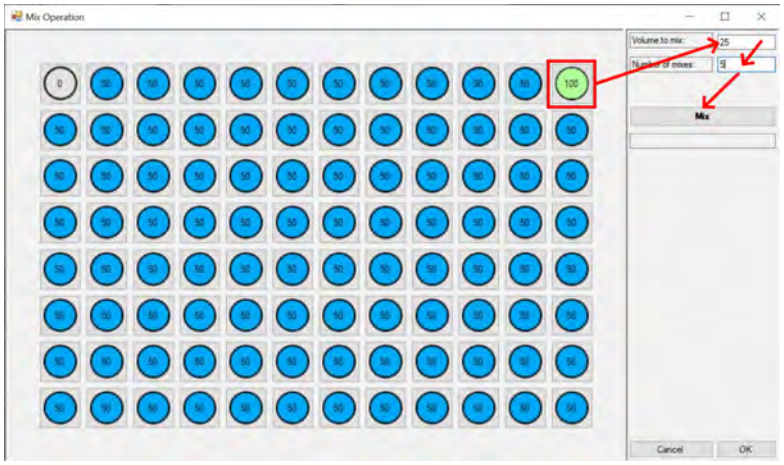


Figure B.33: **25μL will be mixed 5 tips from the well containing 100μL.**

The Mix operation is now confirmed. This means the pump will aspirate then dispense 25μL 5 times into the selected well.

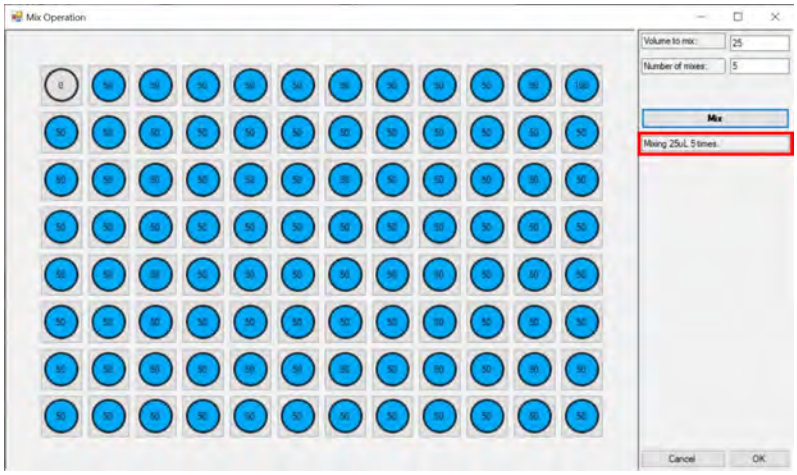


Figure B.34: **The Mix Operation of 25μL is confirmed.**

Click OK and we will see that the mix operation has been successfully added.

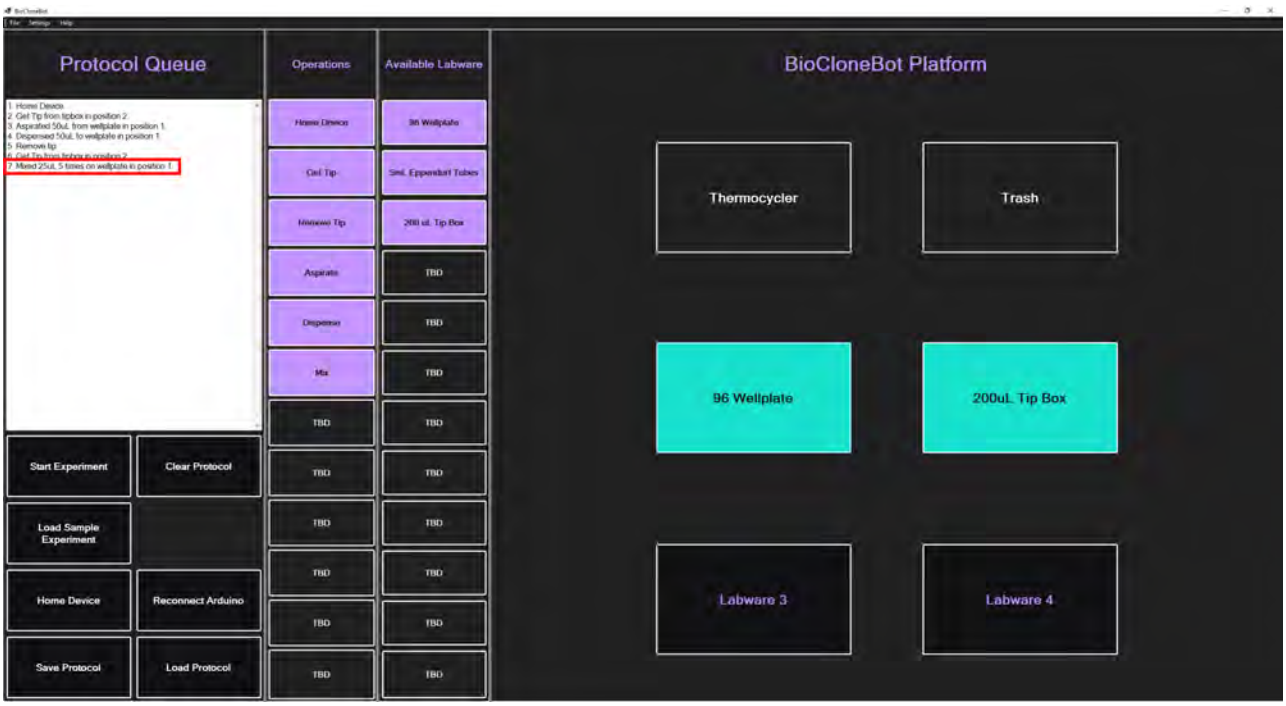


Figure B.35: **The Mix Operation is added to the Protocol Queue.**

Next we will add one final Remove Tip operation. Click Remove Tip to add the final operation.

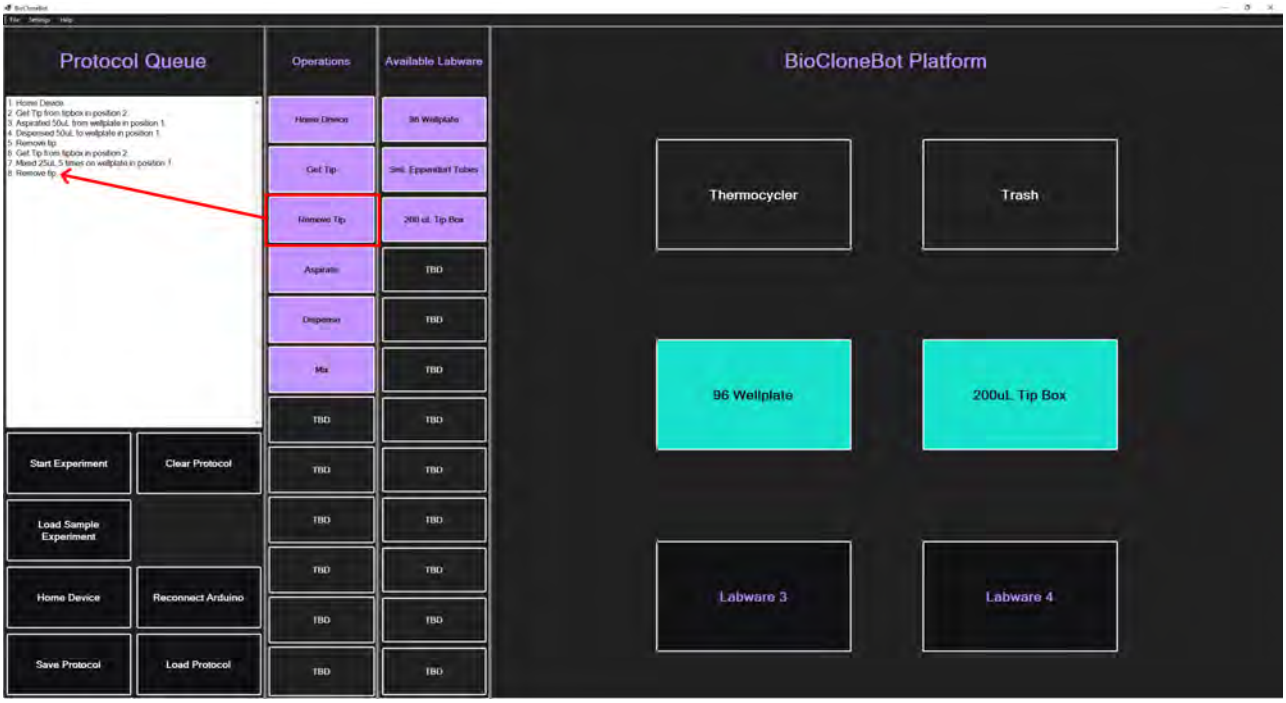


Figure B.36: **Selecting Remove Tip adds a second Remove Tip Operation to the Protocol Queue.**

Our protocol is finally complete.

B.4 Running, Saving, Loading, and Clearing Protocols

Running a Protocol

Now that our experiment has been set up from the previous section, we can add the labware to the platform. Go ahead and load a 96 wellplate into Position 1 and a 200μL tip box into Position 2 on the physical BioCloneBot. Fill the top left well in the wellplate full of water. This will be the water used for the test.

Click the Start Experiment button.

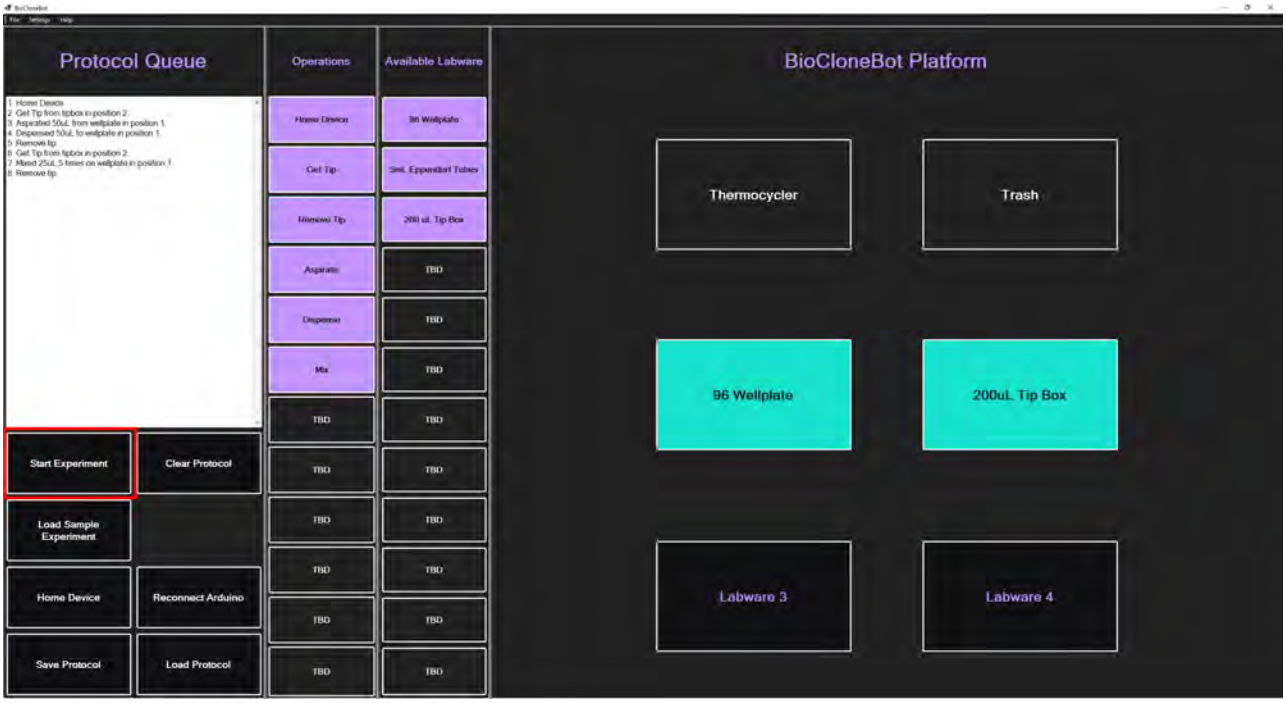


Figure B.37: **Pressing the Start Experiment button will begin the protocol.**

This will result in the following pop-up:

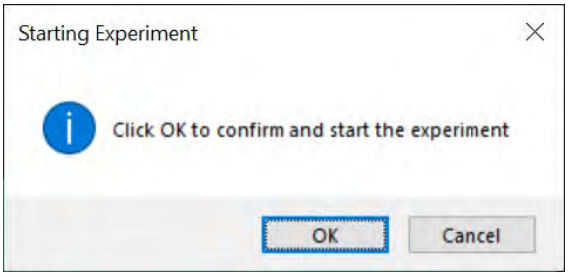


Figure B.38: **Start Experiment confirmation window.**

Once you are sure that your labware and protocol is properly correct, click OK to start the protocol. The front-end generates a list of commands while setting up the protocols. The following loop runs until all commands have been completed:

(1) Send current command to Arduino

(2) Wait for Arduino to send response indicating the completion of the command

(3) Repeat until all commands have been run

Once the experiment is done, we will receive the following pop-up:

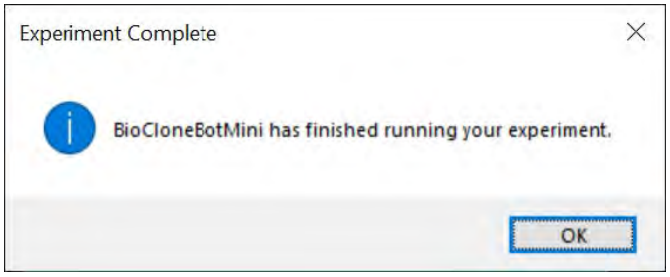


Figure B.39: **Experiment finished pop-up.**

Click OK to finish the experiment.

Saving a Protocol To save time and prevent repeating protocol steps over and over, saving has been implemented.

Once you have finished designing a protocol, click the Save Protocol button in the bottom left.

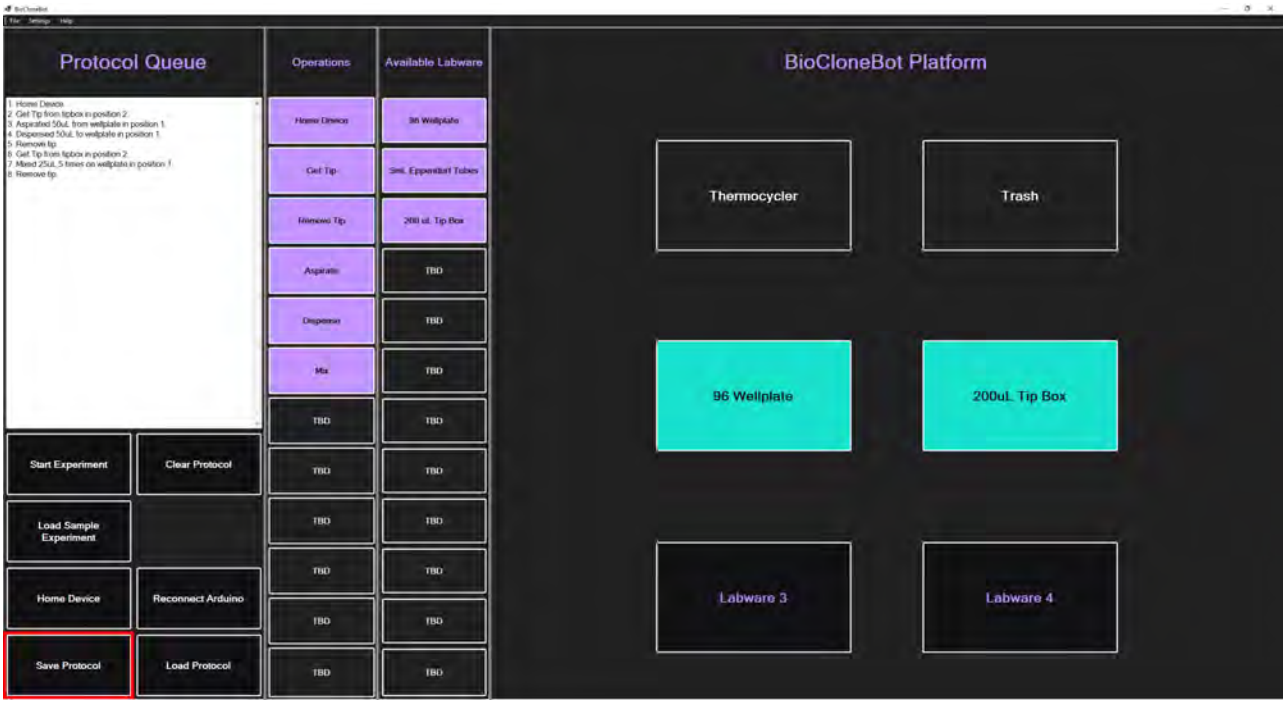


Figure B.40: **Press the Save Protocol button to save your protocol to a JSON file.**

A dialog window will open.

Select your save location, enter in a name for your experiment, and click Save.

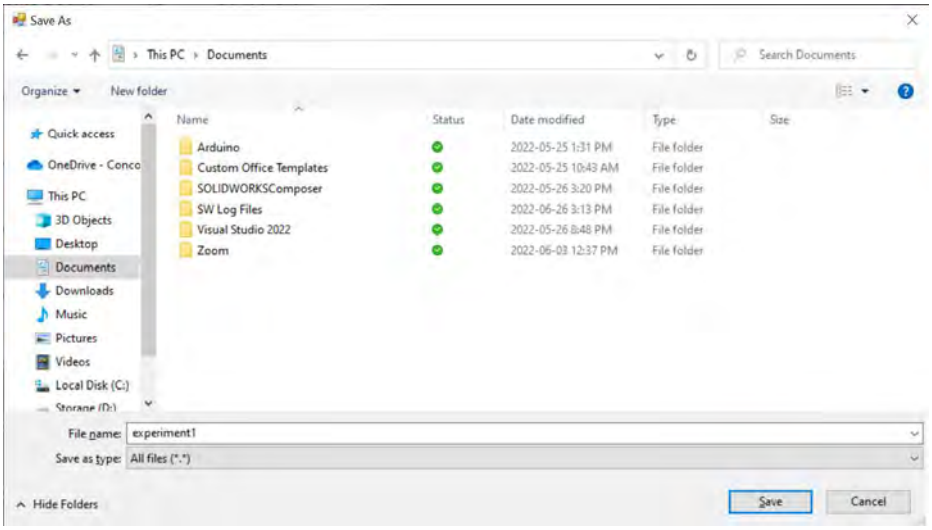


Figure B.41: **Save Protocol Window.**

This will generate a file containing your labware, the protocol, and the final conditions of the labware after all the protocol is run.

Loading a Protocol Click the Load Protocol button in the bottom left.

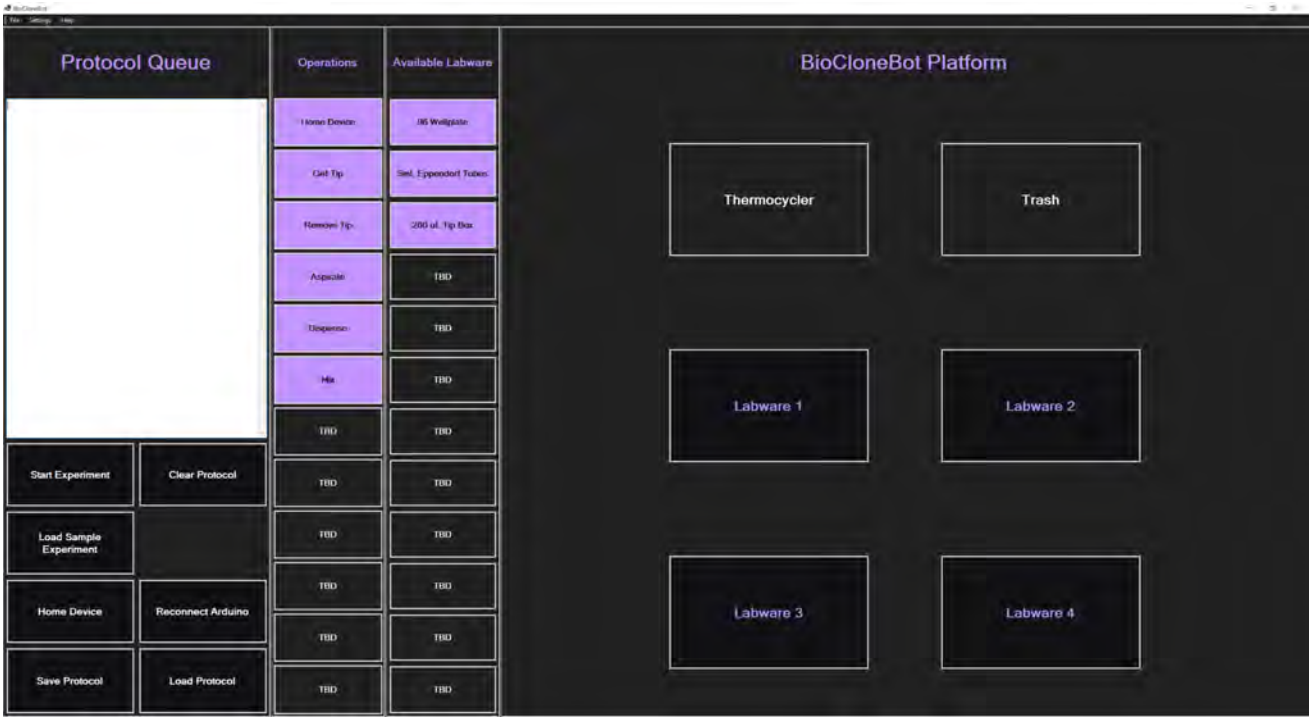


Figure B.42: **Press the Load Protocol button to load a protocol from a JSON file.**

A selection window will open. Navigate to the location of your protocol and press Open. Your protocol will now load into the software.

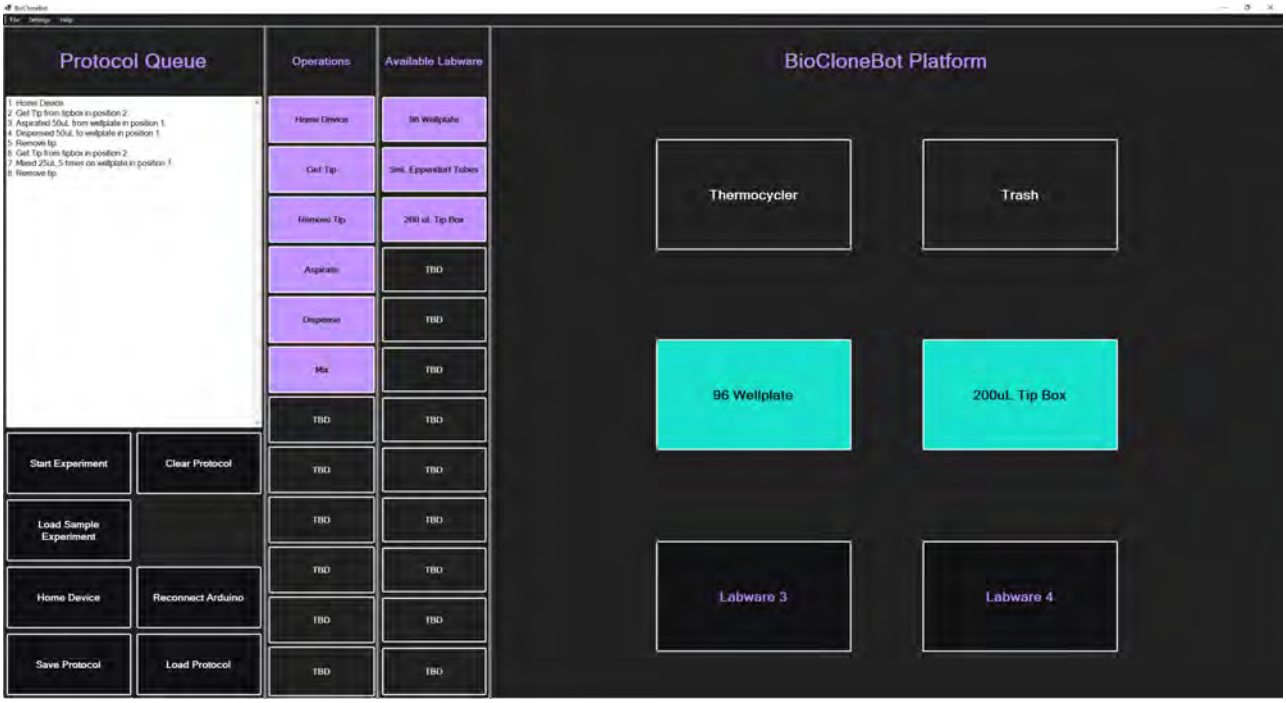


Figure B.43: **The protocol is loaded into the BioCloneBot software.**

Appendix C

Bill of Materials

The assembly guide, software, guide, BOM, and code base can be accessible through the following GitHub repo: BioCloneBot Github

Item	Qty	Price EA (CAD)	Price Total (CAD)	Notes
Ender 3 Pro 3D Printer	1	$326.42	$326.42	Acts as base of the BioCloneBot.
Ender Xtender 400 for Ender 3 Pro V1	1	$247.58	$247.58	
Arduino Mega 2560 R3 Clone	1	$29.95	$29.95	
DRV8825 Stepper Motor Driver	4	$10.76	$10.76	
450W Computer Power Supply	1	$89.99	$89.99	
830 Tie Points Breadboard with 65 Jumper Wires	1	$11.99	$11.99	
1390 Electronic Components Kit	1	$33.86	$33.86	
6 Color, 25ft each 16AWG wire	1	$46.00	$46.00	
400 Step 24V/1.5A NEMA17 Bipolar Stepper Motor	1	$57.15	$57.15	
Straight Lever Limit Switch	1	$3.24	$3.24	
Misumi MTSRA10-100-S30-Q6 Lead Screw	1	$31.79	$31.79	
Misumi MRG-16-5-6 Shaft Coupler	1	$26.57	$26.57	
Misumi MTSNR10 Lead Screw Nut	1	$28.19	$28.19	
Hamilton 50μL Microliter Syringe	1	$110.19	$110.19	Model 7637-01 with removable RN nut

Hamilton 250μL, Microliter Syringe	1	\$145.12	\$145.12	Model 7639-01 with removable RN nut
Two part epoxy	1	\$7.89	\$7.89	This is used for gluing the tip adapter to the syringe nut.
1-5/8" x 3/16" Compression Spring	2	\$2.29	\$4.58	
JST PH Connector Kit	1	\$17.59	\$17.59	Used for stepper motor wires/limit switches.
JST XH Connector Kit	1	\$34.47	\$34.47	Used for stepper motor wires/limit switches.
Soldering Iron Kit	1	\$24.99	\$24.99	
Socket Head Cap Screw Set	1	\$36.99	\$36.99	Try to get a set that comes with various M2, M3, M4, and M5 screws like part #8011655040381 from Ninde-jin
Digital Multimeter	1	\$15.99	\$15.99	
Solderless Crimp Terminal Connector Kit	1	\$26.97	\$26.97	I recommend the Glarks GB007 kit
Crimping Tool	1	\$43.00	\$43.00	Used for making the wires for the stepper motors and limit switches. I highly recommend using the Engineer PA-09 Crimp Tool
2-Terminal On/Off Switch	1	\$2.00	\$2.00	
Terminal Block	1	\$10.00	\$10.00	Use a terminal block that has a minimum of 10 sections and supports a minimum of 30A per section.
18m Roll Electrical Tape	1	\$1.49	\$1.49	For covering soldering work.
			Total Cost	\$1,424.76

Table C.1: **BioCloneBot parts bill of materials.**

Item	**Qty**	**Price EA (CAD)**	**Price Total (CAD)**	**Notes**
Ender 3 Pro 3D Printer	1	\$326.42	\$326.42	One Ender 3 Pro is for 3D printing the parts. The other will be the base of the BioCloneBot.
Black 1.75 mm Standard PLA filament	2 kg	\$49.95	\$49.95	PLA was used for printing all of the BioCloneBot parts.
Soldering Iron Kit	1	\$24.99	\$24.99	
Digital Multimeter	1	\$15.99	\$15.99	

Crimping Tool	1	$43.00	$43.00	Used for making the wires for the stepper motors and limit switches. I highly recommend using the Engineer PA-09 Crimp Tool
			Total Cost	$460.35

Table C.2: **Tools and manufacturing equipment bill of materials**

Appendix D

DNA Assembly Sequences

D.1 Restriction Enzyme Digestion & Ligation Sequences

D.1.1 CEN6/URA3 digested with BsaI

gctggaaatctgctcgtcagtggtgctcacactgacgaatcatgtacagatcataccgatgactgcctggcgactcacaactaagcaagacagccggaac
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D.1.2 GFP digested with BsaI

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D.1.3 Full CEN6/URA3 with GFP dropout ligated

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D.2 Gibson Assembly Sequences

D.2.1 PCR Amplicon CEN6/URA3 Backbone with overhang

gctggaaatctgctcgtcagtggtgctcacactgacgaatcatgtacagatcataccgatgactgcctggcgactcacaactaagcaagacagccggaac
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cgtgagaccgaaagtga

D.2.2 PCR Amplicon GFP Insert with overhang

ttatcccctgattctgtggacaacgtgagaccgaaagtgaaacgtgatttcatgcgtcattttgaacattttgtaaatcttatttaataatgtgtgcggcaattcac
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D.2.3 FWD Primer for CEN6/URA3 Backbone

ttctgcgtttataggtctcagctggaaatctgctcgtcag

D.2.4 REV Primer for CEN6/URA3 Backbone

tcactttcggtctcacgttgtccacagaatcaggggataa

D.2.5 FWD Primer for GFP

ttatcccctgattctgtggacaacgtgagaccgaaagtga

D.2.6 REV Primer for GFP

ctgacgagcagatttccagctgagacctataaacgcagaa

D.2.7 Full Gibson Assembly of CEN6/URA3 with GFP

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D.3 Sequenced Gibson Assembly of CEN6/URA3 with GFP

CTGTACAAATGACCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGT
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CTTTCTGCGTTTATAGGTCTCAGCTGGAAATCTGCTCGTCAGTGGTGCTCACACTGACGAATC
ATGTACAGATCATACCGATGACTGCCTGGCGACTCACAACTAAGCAAGACAGCCGGAACCAG
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D.4 Golden Gate Cloning Sequences

D.4.1 CEN6/URA3 GFP dropout plasmid

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D.4.2 pYTK011 pPGK1 promoter plasmid

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D.4.3 pYTK034 mRuby gene plasmid

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D.4.4 pYTK056 tTDH1 terminator

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D.4.5 Full Golden Gate Assembled pPGK1-mRuby-tTDH1 CEN6/URA3 Plasmid

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